

REMARKS

The Office Action dated April 28, 2005, has been received and reviewed.

Claims 1-27 are currently pending and under consideration in the above-referenced application. Each of claims 1-27 stands rejected.

Reconsideration of the above-referenced application is respectfully requested.

Rejections Under 35 U.S.C. § 102

Claims 1, 2, 5-7, 9, 11, 15, and 21 stand rejected under 35 U.S.C. § 102(b) for reciting subject matter which is purportedly anticipated by that described in Odom, O.W., et al., "An apparent conformational change in tRNA^{Phe} that is associated with the peptidyl transferase reaction," *Biochemie* 69:925-38 (1987) (hereinafter "Odom").

A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single reference which qualifies as prior art under 35 U.S.C. § 102. *Verdegaal Brothers v. Union Oil Co. of California*, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987). The identical invention must be shown in as complete detail as is contained in the claim. *Richardson v. Suzuki Motor Co.*, 9 USPQ2d 1913, 1920 (Fed. Cir. 1989).

With respect to inherency, M.P.E.P. § 2112 provides:

The fact that a certain result or characteristic may occur or be present in the prior art is not sufficient to establish inherency of that result or characteristic. *In re Rijckaert*, 9 F.3d 1531, 1534, 28 USPQ2d 1955, 1957 (Fed. Cir. 1993) . . . 'To establish inherency, the extrinsic evidence 'must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill . . . ' *In re Robertson*, 169 F.3d 743, 745, 49 USPQ2d 1949, 1950-51 (Fed. Cir. 1991).

Independent claim 1 is drawn to a biomolecular substrate that includes a core molecular backbone and first and second dyes that are covalently attached to the core molecular backbone. When the biomolecular substrate is not covalently modified, the first and second dyes associate to form a quenched intramolecular dye dimer. Such quenching is effected at least in part through a non-fluorescence resonance energy transfer (non-FRET) mechanism. When the biomolecular

substrate is covalently modified, the first and second dyes dissociate, which results in quenching of at least one of the dyes.

Independent claim 15 recites a method for assaying covalently biomolecular modification. The method of independent claim 15 includes providing a sample that includes a core molecular backbone, as well as first and second dyes covalently attached to the core molecular backbone. Non-FRET induced changes in fluorescent or absorbance characteristics of the biomolecular substrate may be quantified to determine whether or to the biomolecular substrate has been covalently modified.

Non-FRET mechanisms for inducing changes in fluorescence or absorbance characteristics of a fluorescent dye include so-called “static quenching,” or “ground state quenching,” interactions between the fluorescent dye and another molecule (*e.g.*, another dye), which is known in the art as a “quencher.” Ground state quenching includes physical interaction, such as binding, between a fluorescent dye molecule, or fluorophore, and a quencher to form a nonfluorescent complex between the fluorophore and the quencher. Binding of the quencher to the fluorophore frequently perturbs the absorption spectrum of the fluorophore. Moreover, upon absorbing light, this non-FRET, nonfluorescent complex immediately returns to the ground state without emitting a photon. *See, e.g.*, The Glen Report, Vol. 17, No. 1, September 2004, a copy of which is enclosed for the sake of convenience (hereinafter “the Glen Report”).

FRET is known to those of ordinary skill in the art to be fundamentally different from non-FRET mechanisms, such as ground state quenching. Specifically, FRET is a photophysical process in which a first fluorophore, which is commonly referred to as a “donor molecule,” is excited and, thus, fluoresces. The fluorescence, or energy, emitted by the donor molecule is then absorbed by an “acceptor molecule.” The amount of quenching that occurs as the acceptor molecule absorbs energy from the donor molecule depends on a variety of factors, including the overlap of the emission spectrum of the donor molecule and the absorption spectrum of the acceptor molecule, the quantum yield of the donor molecule, the relative orientations of the donor and acceptor molecules, and the physical distance between the donor and acceptor molecules. *See, e.g.*, the Glen Report and [http://etd/caltech.edu/etd/available/etd-05112004-101833/unrestricted/\(06\)ch4_Title_text.pdf](http://etd/caltech.edu/etd/available/etd-05112004-101833/unrestricted/(06)ch4_Title_text.pdf), titled “Chapter 4: Fluorescence Resonance Energy

Transfer (FRET) by Minor Groove-Associated Cyanine-Polyamide Conjugates,” published May 11, 2004, a copy of which is enclosed.

Thus, non-FRET quenching may occur due to the formation of a complex between a fluorophore and a quencher, whereas FRET quenching occurs without binding of a fluorophore and a quencher. Furthermore, the quencher of a non-FRET quenching pair of molecules causes perturbations in the absorption spectrum of the fluorophore, whereas, in FRET quenching, the acceptor molecule does not perturb the absorption spectrum of the donor molecule (*i.e.*, the fluorophore).

Moreover, when a ground state quenching complex has been formed, there may be no emission by the initially excited fluorophore (*i.e.*, the “donor molecule” of a FRET pair); thus, non-FRET quenching does not necessarily involve FRET.

The description of Odom relates to use of a pair of fluorescent molecules to detect conformational changes in tRNA^{Phe}. In particular, the methods that are described in Odom included evaluation of tRNA^{Phe} molecules that were labeled with both DCCH (coumarin) and FITC (fluorescein), where DCCH is the donor and FITC is the acceptor in a system in which “energy transfer by donor quenching” occurred. Odom, page 931. Thus, the description of Odom is clearly limited to a biomolecular substrate that is labeled with dyes that are quenched through FRET, as well as to an assay method that includes use of a FRET-quenched dye pair.

Further, as the disclosure of Odom is limited to FRET quenching, and ground state quenching does not necessarily involve FRET, Odom does not inherently describe a biomolecular substrate that is configured for non-FRET quenching or a method in which non-FRET quenching may occur.

Therefore, Odom does not anticipate either the biomolecular substrate of independent claim 1 or the assay method of independent claim 15. Accordingly, under 35 U.S.C. 102(b), the subject matter recited in both independent claim 1 and independent claim 15 is allowable over the subject matter described in Odom.

Each of claims 2, 5-7, 9, and 11 is allowable, among other reasons, for depending directly from claim 1, which is allowable.

Claim 21 is allowable, among other reasons, for depending directly from claim 15, which is allowable.

Withdrawal of the 35 U.S.C. § 102(b) rejections of claims 1, 2, 5-7, 9, 11, 15, and 21 is respectfully solicited.

Rejections Under 35 U.S.C. § 103(a)

Claims 1-27 stand rejected under 35 U.S.C. § 103(a).

The standard for establishing and maintaining a rejection under 35 U.S.C. § 103(a) is set forth in M.P.E.P. § 706.02(j), which provides:

To establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based on applicant's disclosure. *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991).

Blumenthal in View of Odom and Tyagi

Claims 1-27 stand rejected under 35 U.S.C. § 103(a) for reciting subject matter which is assertedly unpatentable over that taught in Blumenthal, D.K., "Development and Characterization of Fluorescently-Labeled Myosin Light Chain Kinase Calmodulin-Binding Domain Peptides," Molecular and Cellular Biochemistry, 127:128: 45-50 (1993) (hereinafter "Blumenthal"), Odom, and either WO 97139008 of Tyagi or U.S. Patent 6,150,097 to Tyagi (hereinafter "Tyagi").

Blumenthal teaches biomolecular substrates that include a single dye (acrylodan) for measuring changes in the conformation of a calmodulin binding domain of a peptide when the peptide binds calmodulin. In one of the methods, the analyzed peptide included naturally occurring tryptophan residues and was employed as a FRET donor molecule, while the acrylodan

acted as the FRET acceptor molecule. When calmodulin binds noncovalently to the peptide, a conformational change in the peptide occurred, which decreased the distance between the tryptophan and the acrylodan and, thus, caused an increase in FRET quenching.

The teachings of Odom have been summarized above. Most notably, the teachings of Odom are limited to use of a dye pair in which changes in FRET quenching are indicative of conformational changes in tRNA^{Phe} that may occur as a the phenylalanine molecule that is carried by the tRNA^{Phe} molecule forms a peptide bond with another amino acid.

Tyagi teaches the use of “Molecular Beacon” oligonucleotide probes to monitor binding of oligonucleotide probes labeled with two fluorescent dyes or a fluorophore and a quencher to target sequences in nucleic acids. While fluorescence of the fluorophore may be quenched when the quencher “touches” the fluorophore and, thus, ground state quenching may occur, the quenching is merely indicative of hybridization reactions between nucleic acids, not of covalent modification of the labeled nucleic acid probe.

It is respectfully submitted that the teachings of Blumenthal, Odom, and Tyagi do not support a *prima facie* case of obviousness against any of claims 1-27 for a number of reasons.

First, none of these references teaches or suggests each and every element of any of claim 23 or 24. Independent claim 23 is allowable since none of Blumenthal, Odom, or Tyagi teaches or suggest a method for assaying protein kinase activity, let alone various aspects of that method, including provision of a biomolecular substrate that includes a KID peptide sequence or a pair of molecules that, when the biomolecular substrate is not covalently modified, form an intermolecular dye dimer, but when the biomolecular substrate is phosphorylated, dissociate to reduce quenching between the pair of molecules. Claim 24 is allowable, among other reasons, for depending directly from claim 23, which is allowable.

Second, without improperly relying upon the disclosure of the above-referenced application, one of ordinary skill in the art would not have been motivated to combine teachings from Blumenthal, Odom, and Tyagi in the manner that has been asserted. This is because none of the references provides any motivation for using non-FRET quenching techniques with a core molecular backbone to detect covalent changes in the core molecular backbone. More specifically, while it is acknowledged that tRNA molecules undergo a conformational change

during the peptidyl transferase reaction, Odom actually dissuades from the asserted combination of teachings by indicating that the FRET-based fluorescence quenching results set forth therein “do not conclusively establish that tRNA undergoes a conformational change . . . during the peptidyl transferase reaction.” Odom, Abstract. Due to this admitted unreliability, it does not appear that one of ordinary skill in the art would have any reason to supply a purportedly analogous technology (non-FRET quenching) to the methodology that is taught in Odom. Therefore, Odom provides no motivation for one of ordinary skill in the art to use substitute non-FRET quenching techniques for the FRET quenching techniques disclosed therein.

As the teachings of Blumenthal, Odom, and Tyagi do not support a *prima facie* case of obviousness, it is respectfully submitted that, under 35 U.S.C. § 103(a), the subject matter recited in each of claims 1-27 is allowable over the teachings of Blumenthal, Odom, and Tyagi.

Macala, Shultz, Blumenthal, and Odom or Tyagi

Claims 1-27 stand rejected under 35 U.S.C. § 103(a) for reciting subject matter that is allegedly unpatentable over the subject matter taught in Macala, L.J., et al., “Measurement of cAMP-Dependent Protein Kinase Activity Using a Fluorescent-Labeled Kemptide,” *Kidney International*, 54: 1746-50 (1998) (hereinafter “Macala”), U.S. Patent 5,580,747 to Schultz et al. (hereinafter “Schultz”), or Ventura, C., et al., “Phorbol Ester Regulation of Opioid Peptide Gene Expression in Myocardial Cells,” *The Journal of Biological Chemistry*, 270(50): 301 15-20 (1995) (hereinafter “Ventura”), in view of teachings from Blumenthal and Odom or Tyagi.

It is respectfully submitted that none of these references, taken separately or in any combination, supports a *prima facie* case of obviousness against any of claims 1-27.

With respect to the purported combination of teachings from Macala, Shultz, Blumenthal, and Odom, it is respectfully submitted that none of these references teaches or suggests a biomolecular substrate that includes a core molecular backbone and a pair of dyes that undergo ground state interactions prior to covalent modification of the backbone and dissociate from one another following covalent modification of the backbone. Moreover, none of these references

teaches or suggests use of such a biomolecular substrate in an assay. Thus, none of these references teaches or suggests each and every element of any of claims 1-27.

In addition, since Macala, Shultz, Blumenthal, and Odom, taken together, do not supply each and every element of any of claims 1-27, one of ordinary skill in the art would have no reason to expect the purported combination of teachings from these references to be successful.

Moreover, for the foregoing reasons, it is respectfully submitted that, without improperly relying upon the hindsight provided by the disclosure and claims of the above-referenced application, one of ordinary skill in the art would have had no reason to combine the teachings of Macala, Shultz, Blumenthal, and Odom in the manner that has been asserted.

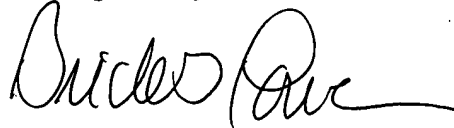
As for the asserted combination of teachings from Macala, Shultz, Blumenthal, and Tyagi, it is respectfully submitted that one of ordinary skill in the art would have had no reason to combine the teachings of these references. Of these references, only Tyagi teaches fluorescence quenching between a pair of dye molecules. The teachings of Tyagi are limited to reductions in fluorescence quenching as a single stranded probe nucleic acid hybridizes with another single stranded nucleic acid, which is a noncovalent modification of the probe nucleic acid. While Macala, Shultz, and Blumenthal merely teach use of single or non-interacting fluorescent molecules on a core molecular substrate. None of Macala, Shultz, or Blumenthal teaches or suggests that a pair of dyes may be used to indicate covalent changes to a core molecular substrate. Thus, without improperly relying upon the hindsight provided by the disclosure and claims of the above-referenced application, it is not seen how the teachings of any of these references could have provided one of ordinary skill in the art to use the dye pair of Tyagi to detect covalent modifications to a core molecular substrate.

As no combination of teachings from Macala, Shultz, Blumenthal, and Odom or Tyagi supports a *prima facie* case of obviousness under 35 U.S.C. § 103(a), it is respectfully submitted that, under 35 U.S.C. § 103(a), the subject matter recited in each of claims 1-27 is allowable over the teachings of these references.

CONCLUSION

It is respectfully submitted that each of claims 1-27 is allowable. An early notice of the allowability of each of these claims is respectfully solicited, as is an indication that the above-referenced application has been passed for issuance. If any issues preventing allowance of the above-referenced application remain which might be resolved by way of a telephone conference, the Office is kindly invited to contact the undersigned attorney.

Respectfully submitted,

A handwritten signature in black ink, appearing to read "Brick G. Power", with a long horizontal flourish extending to the right.

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Date: July 28, 2005
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Chapter 4

**Fluorescence Resonance Energy Transfer (FRET)
by Minor Groove-Associated Cyanine-Polyamide Conjugates**

The work described in this chapter was accomplished in collaboration with V. Rucker (Dervan group; Caltech).

Abstract

Fluorescence resonance energy transfer has been used for both qualitative and quantitative analysis of nucleic acid structure in biologically relevant contexts. The timescale of FRET and the range of distances (10-90 Å) over which it is useful are complementary to other techniques commonly applied to the analysis of DNA. Previous work in the Dervan group has attempted to use of FRET between fluorophore-polyamide conjugates as a means of sequence specific detection of DNA; however, these efforts were unsuccessful, possibly resulting from the choice of fluorophores and the design of polyamide conjugates.

This chapter describes FRET between cyanine-hairpin polyamide conjugates in a model system. The physical properties of cyanine conjugates, including their spectral characteristics, affinities for DNA, and the degree of quenching they exhibit in the absence of DNA, are summarized. Preliminary results using a Cy3 fluorophore as the donor and a Cy5 fluorophore as the acceptor are presented in detail. Alternative donors and acceptors, previously synthesized, are suggested for use in future experiments. Potential applications for polyamide-based FRET are also presented.

Background and Significance

Fluorescence resonance energy transfer (FRET) is a spectroscopic process by which a donor fluorophore transfers energy nonradiatively over long distances (10-90 Å) to an acceptor fluorophore. The relationship between fluorophore distance and energy transfer was first described by Förster in the 1940's,¹ and was later verified by Stryer and colleagues, in the late 1970's.² A number of advantages are inherent to this technique, including the sensitivity of fluorescence-based detection, the relatively short timescale of energy transfer, and the appreciable range of distances over which it can be applied.³

The molecular processes underlying FRET have been reviewed extensively and are illustrated in Figure 4.1.^{4,5} The first step involves absorption of energy by the donor molecule, resulting in excitation from the ground state, S_0^D , to an excited singlet state, S_1^D . Several excited states are available to the donor; however, vibrational relaxation to S_1^D by internal conversion is rapid, ensuring that a majority of emission occurs from this state. Several fates are possible for the excited donor, including spontaneous emission and nonradiative processes. If a suitable acceptor fluorophore is nearby, then nonradiative energy transfer between the donor and acceptor can occur. This transfer involves a resonance between the singlet-singlet electronic transitions of the two fluorophores, generated by coupling of the emission transition dipole moment of the donor and the absorption transition dipole moment of the acceptor. Thus, the efficiency of FRET and the range of distances over which it can be observed are determined by the spectral properties of a given donor-acceptor pair.

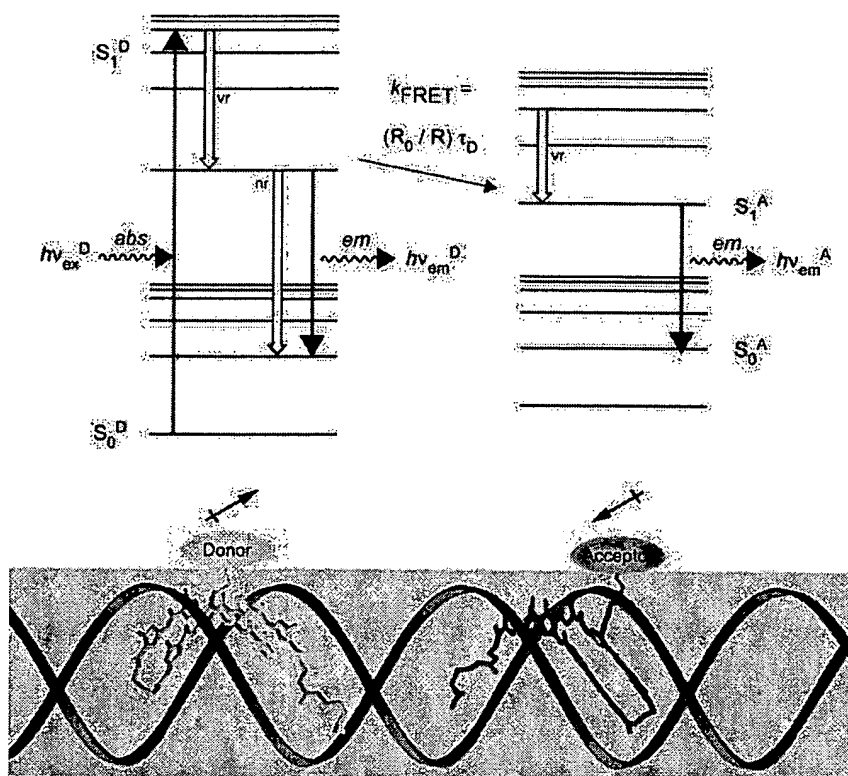


Figure 4.1 Molecular processes underlying FRET illustrated for fluorophore-polyamide conjugates.

FRET can be experimentally measured in a number of ways, using either time-resolved or steady-state techniques. The most commonly employed methods for quantifying FRET monitor either the reduction in the donor quantum yield in the presence of acceptor or the enhancement of acceptor emission in the presence of donor. Quantum mechanics dictates that the rate of energy transfer correlates with the inverse sixth power of the distance separating the fluorophores, R . In the context of steady-state experiments, this relationship allows the efficiency of energy transfer, E_T , to be translated into relative distances according to Equation 1, where

$$E_T = 1 / \left[1 + \left(\frac{R}{R_0} \right)^6 \right]$$

R_0 is the characteristic Förster radius for a given donor-acceptor pair. The Förster radius is defined by Equation 2, where Φ_D is the fluorescent quantum yield of the donor in the absence of acceptor, κ is an orientation factor that depends on relative dipole positions of the donor and acceptor, η is the refractive index of the medium, and $J(\lambda)$ is the spectral overlap of donor emission and acceptor absorption. Thus, when $R = R_0$ the efficiency of FRET is 50%. In practice, a more manageable version

$$R_0^6 = 8.8 \times 10^{-28} \cdot \Phi_D \cdot \kappa^2 \cdot \eta^{-4} \cdot J(\lambda)$$

of this relationship is desirable, using the definition of quantum yields to derive Equation 3 which correlates two experimentally determined values, E_T and Φ_D , with the distance between two donor and acceptor.

$$E_T = 1 - \left(\Phi_{DA} / \Phi_D \right)$$

FRET can be used as either a quantitative tool for determining absolute distances in macromolecular assemblies or for quantitative measure of relative distances.⁶ In the context of polyamide-based FRET, quantitative techniques present several experimental difficulties and offer no real advantage to existing sequencing techniques. Qualitative applications of FRET, on the other hand, are well-suited to polyamides and can provide structural insight for DNA and DNA-protein complexes. Similar approaches to FRET have been used to measure DNA bending and kinking inherent to purine tracts or resulting from protein association.^{7,8} FRET can also be combined with other analytical techniques, including HPLC, flow cytometry, or gel electrophoresis to allow greater S/N ratios.

Previous Work toward Polyamide-Based FRET (Figure 4.2)

Seminal efforts to demonstrate FRET between fluorophore-polyamide conjugates bound proximally in the minor groove were carried out by Scott Carter. Eight-ring hairpin polyamides, bearing coumarin or BODIPY moieties at the turn position, were synthesized and screened using a set of model oligonucleotide duplexes, without success. The relatively poor specificity of the polyamide scaffold selected for donor attachment, **1**, was discussed in Chapter 3 and must be considered at least a contributing factor to the experimental failure. The choice of the turn for covalent modification with fluorophores is also suspect as similar conjugates have shown either reduced affinity or variation in fluorescence with respect to the proximal DNA sequences. The fluorophores selected in these experiments are also characterized by lower quantum yields and molar extinction coefficients than their cyanine counterparts. Finally, the oligonucleotide duplexes employed to measure FRET examined extremely short inter-fluorophore distances which allows energy transfer to occur by alternate mechanisms (Dexter),⁹ complicating the interpretation of results, if allowing association of both polyamides at all.

Subsequent research efforts, conducted by Victor Rucker, replaced the above fluorophores with the well characterized fluorescein-TMR donor-acceptor pair. A different covalent modification strategy, using the N-Me position of Py residues for fluorophore attachment, was also employed. Oligonucleotide duplexes were redesigned to provide greater fluorophore separations, however, the promiscuous donor-polyamide scaffold, **3**, was maintained and FRET was not observed. A third

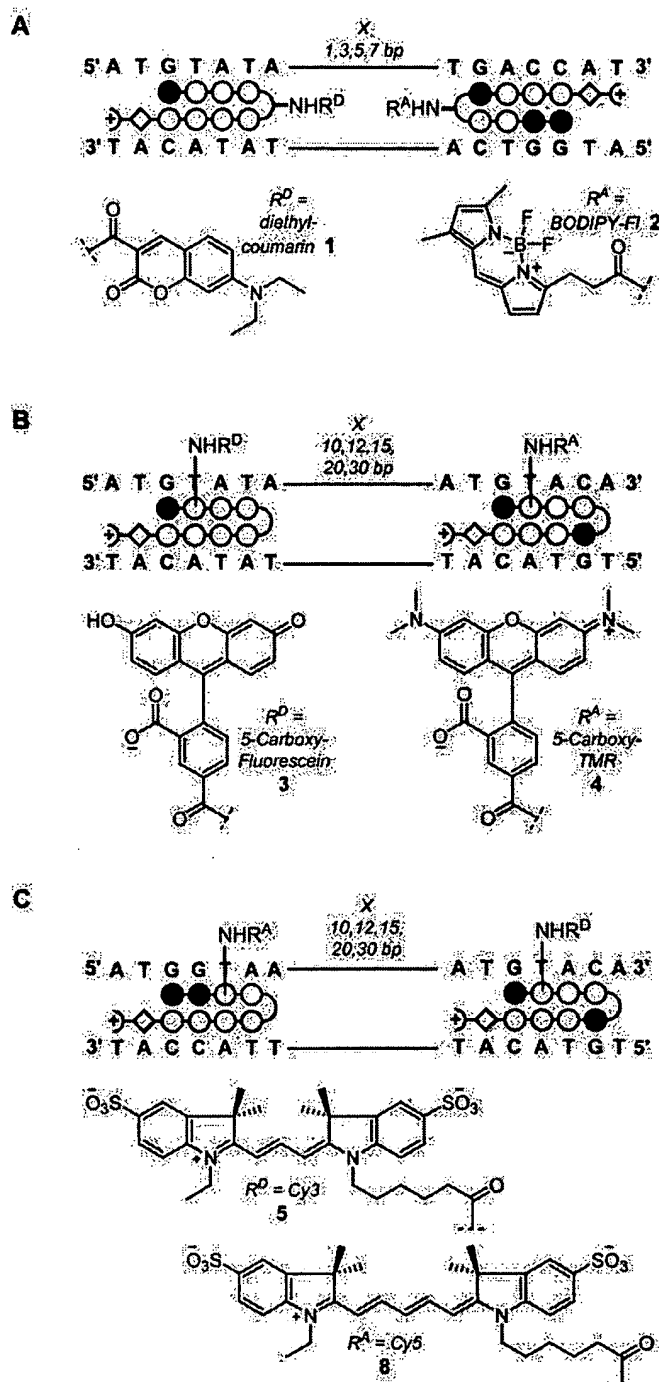


Figure 4.2 Previous efforts to demonstrate polyamide-based FRET. **(A)** First generation conjugates for FRET employed coumarin- and BODIPY-modified turn moieties. **(B)** Second generation conjugates contained Py ring-attached fluorescein and tetramethylrhodamine probes. **(C)** Current polyamide conjugates for FRET use Py ring-attached cyanine probes.

evolution of polyamide-based FRET was then undertaken, using polyamide scaffolds

with greater specificity in conjunction with brighter cyanine probes.

Model System for Polyamide-Based FRET

Oligonucleotide duplexes containing donor- and acceptor-polyamide binding sites separated by variable intervening sequences were carefully designed to avoid the presence multiple binding sites for either polyamide (Figure 4.3A). Fluorophore-fluorophore distances were then determined using the double helix as a spectroscopic ruler (10 bp ~ 34Å). In light of the findings in Chapter 3, hairpin oligonucleotides were used to assess the behavior of cyanine-polyamide conjugates in the presence and absence of DNA (Figure 4.3B). In particular, the affinity of the donor for the acceptor binding site and the affinity of the acceptor for the donor binding site were investigated. Specificity was also addressed in the design of polyamide scaffolds and the promiscuous scaffold used in previous experiments was replaced by a more specific polyamide whose match site is a double base pair mismatch for the other polyamide. Cyanine probes were attached to these polyamides using the Py ring as a point of attachment with the aim of minimizing the influence of fluorophores on the DNA recognition properties of the polyamides. A family of hairpin conjugates containing three different donors and two acceptors were synthesized, though FRET experiments to date have focused on the Cy3-Cy5 (5-8) donor-acceptor pair (Figure 4.4).

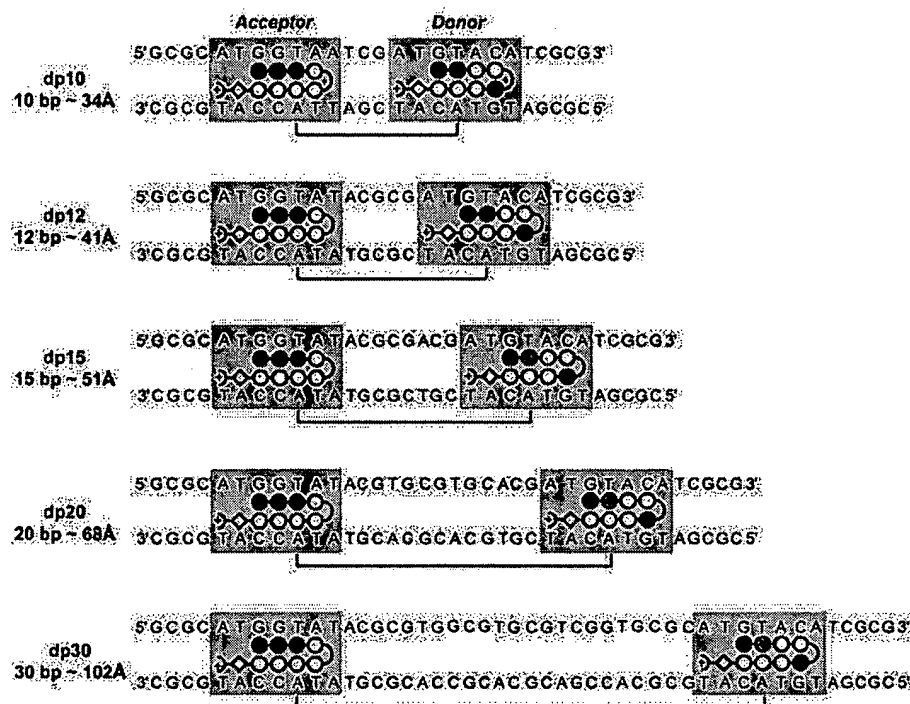
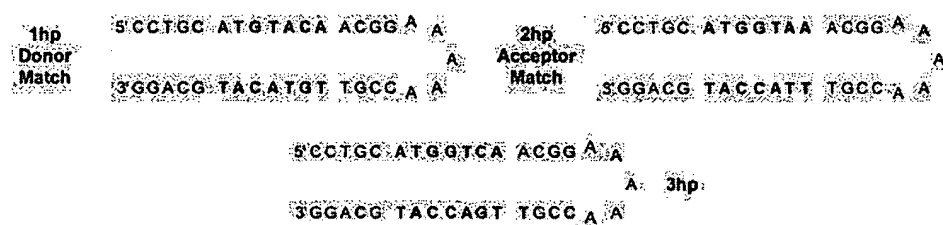
A**B**

Figure 4.3 Model systems for examination of FRET by polyamide conjugates. **(A)** Duplex oligonucleotides for distance-dependent titrations. Circle shaded in blue represents pyrrole ring with attached Cy5 acceptor. Circle shaded in red represents a pyrrole ring with attached Cy3 donor. Inter-fluorophore distances determined using approximation that 10 bp ~ 34 Å. **(B)** Hairpin duplex oligonucleotides used to determine quenching of cyanine-polyamide conjugates.

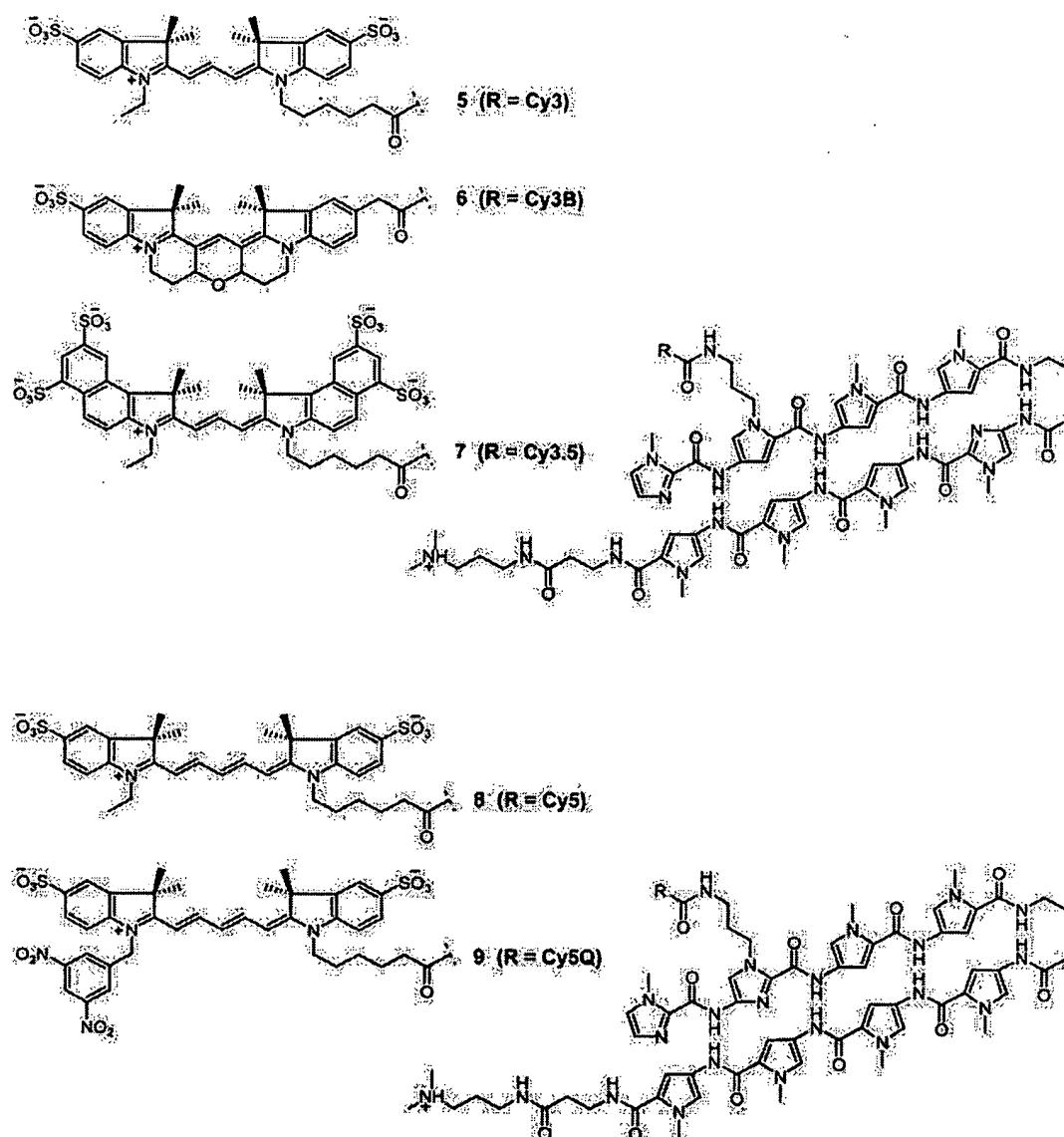


Figure 4.4 Chemical structures of cyanine-polyamide conjugates.

Physical Properties of Cyanine-Polyamide Conjugates

The cyanine donors employed, Cy3 (**5**), Cy3B (**6**), and Cy3.5 (**7**) span a range of excitation-emission wavelengths complementary to the Cy5 acceptor (**8**), as evidenced by their normalized absorption-emission spectra (Figure 4.5). These compounds have intrinsically higher molar extinction coefficients than TMR or

fluorescein and show no absorption in the 300 nm range, allowing more reliable determination of conjugate concentration by spectroscopic methods (Table 4.1). The affinities of cyanine conjugates for their match sites were determined by DNase I footprinting and, with the exception of **7**, show reasonable association constants (Table 4.1). The Cy3.5 chromophore contains four sulfate groups that could generate unfavorable electronic contacts with the phosphate backbone of the DNA upon polyamide association in the minor groove.

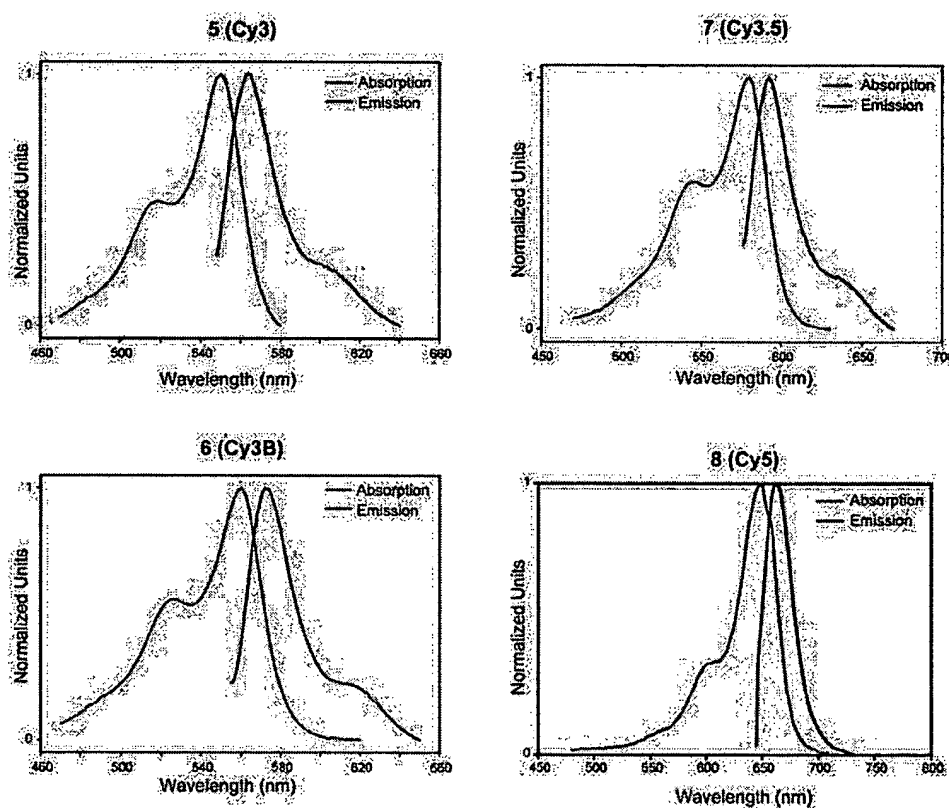


Figure 4.5 Normalized absorption emission spectra for cyanine-polyamide conjugates. All measurements taken in the presence of match duplex DNA.

Table 4.1 Physical properties of cyanine-hairpin polyamide conjugates.

polyamide	λ_{ex}	ϵ ($\text{M}^{-1} \text{cm}^{-1}$)	λ_{em}	K_A (M^{-1})
5	555 nm	75,000	564 nm	1.0×10^9 (5'-AGTACT-3')
6	566 nm	113,000	572 nm	7.5×10^8 (5'-AGTACT-3')
7	585 nm	96,000	592 nm	$< 1.0 \times 10^7$ (5'-AGTACT-3')
8	653 nm	127,000	660 nm	1.0×10^6 (5'-TGGTAA-3')

The occurrence of fluorescence quenching in cyanine conjugates was investigated by titration with hairpin oligonucleotides containing the donor-polyamide binding site, the acceptor-polyamide binding site, and a third polyamide binding site. The conjugates showed different degrees of fluorescent enhancement in the presence of match duplex DNA, increasing in the order: Cy3 < Cy3.5 < Cy5 < Cy3B ~ 5-TMR (Figure 4.6). The Cy3B fluorophore is especially promising, exhibiting comparable fluorescent enhancement to 5-TMR with substantially higher overall intensities.

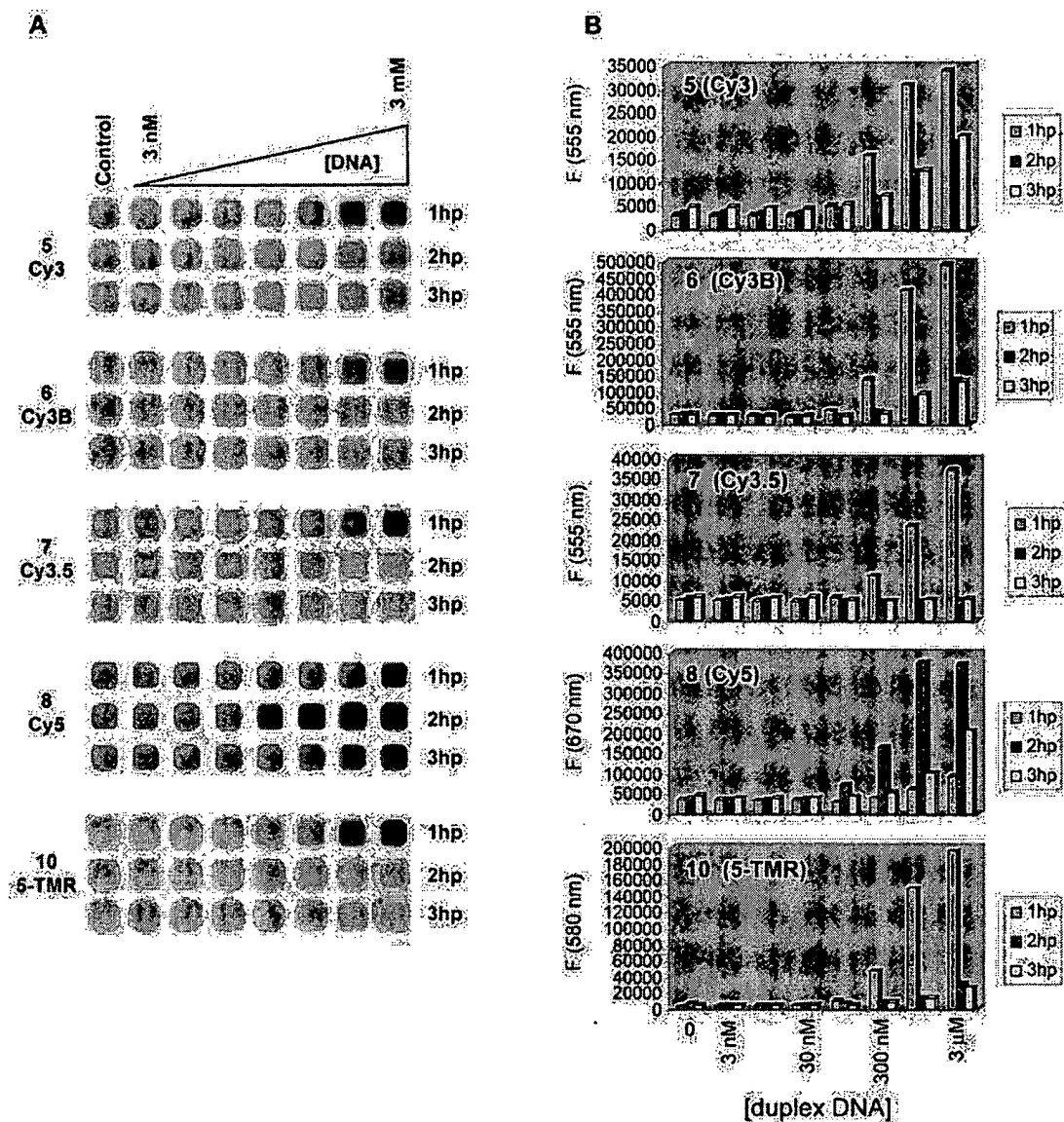


Figure 4.6 Cyanine-polyamide conjugates exhibit fluorescent enhancement when bound to DNA. **(A)** Microplate titration of polyamide conjugates with hairpin oligonucleotides. Cy3, Cy3.5, and Cy3B emission were monitored at 555 nm while 5-TMR fluorescence was monitored at 580 nm, both with excitation at 532 nm. Cy 5 emission was measured at 670 nm with excitation at 633 nm. **(B)** Averaged data from replicate microplate assays is illustrated by plotting fluorescent intensity as a function of duplex concentration.

FRET Experiments Using Cyanine-Hairpin Polyamide Conjugates

Resonance energy transfer by the Cy3-Cy5 (5-8) donor-acceptor pair was observed by steady-state techniques, using both fluorimeter- and microplate-based

assays. The well-separated emission and absorption wavelengths of **5** and **8** allow resonance energy transfer to be measured with respect to donor quenching or sensitized emission of the acceptor (Figure 4.7A). Titration of cyanine-polyamide conjugates **5** and **8** (2 μM) with model oligonucleotide duplexes (Figure 4.3A) resulted in a distance-dependent decrease in the quantum yield of donor **5**. Triplicate measurements in a standard fluorimeter were fitted using Equation 3, giving an observed R_0 of 35.9Å. Similar experiments using microplates gave comparable results.

Subsequent microplate assays with the optimal model duplex (**d10**) examined the influence of concentration and equilibration time on both donor quenching and sensitized emission (Figure 4.8). Polyamide concentrations ranging from 100 nM to 2 μM gave R_0 values comparable to those obtained previously. Higher concentrations (500 nM) were required to facilitate detection of sensitized emission as indicated by S/N ratios, determined relative to a control lacking duplex DNA. Decreasing equilibration times for polyamide conjugates with duplex DNA did not significantly influence transfer efficiencies and this finding was confirmed by DNase I footprinting studies of **8**, which exhibited comparable association constants at both short and long time points.

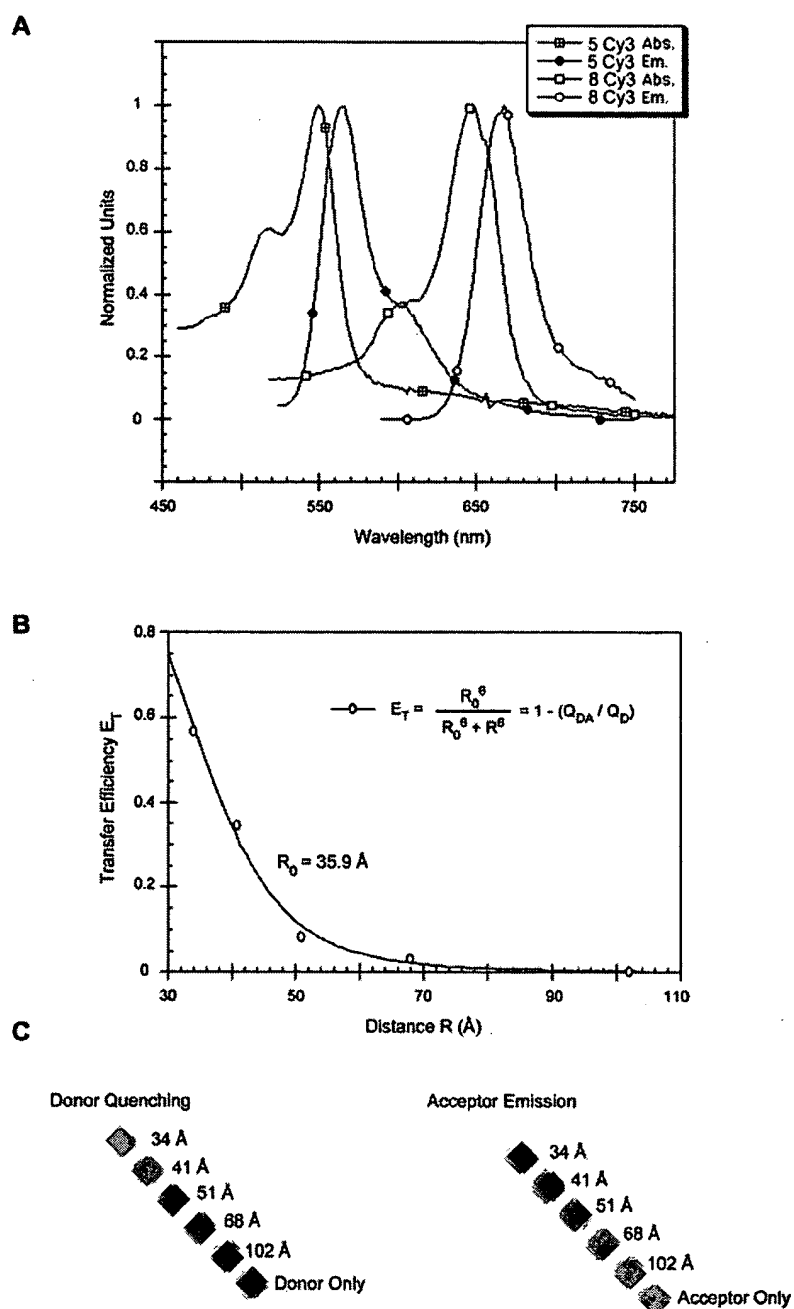


Figure 4.7 Demonstration of FRET by minor groove-associated cyanine-polyamide conjugates. **(A)** Conjugates 5 and 8 show significant spectral overlap. **(B)** Fit of data from duplex oligonucleotide titration shows distance-dependence inherent to resonance energy transfer. **(C)** Microplate assays can monitor FRET by quenching of donor (*left*) and by sensitized emission of acceptor (*right*).

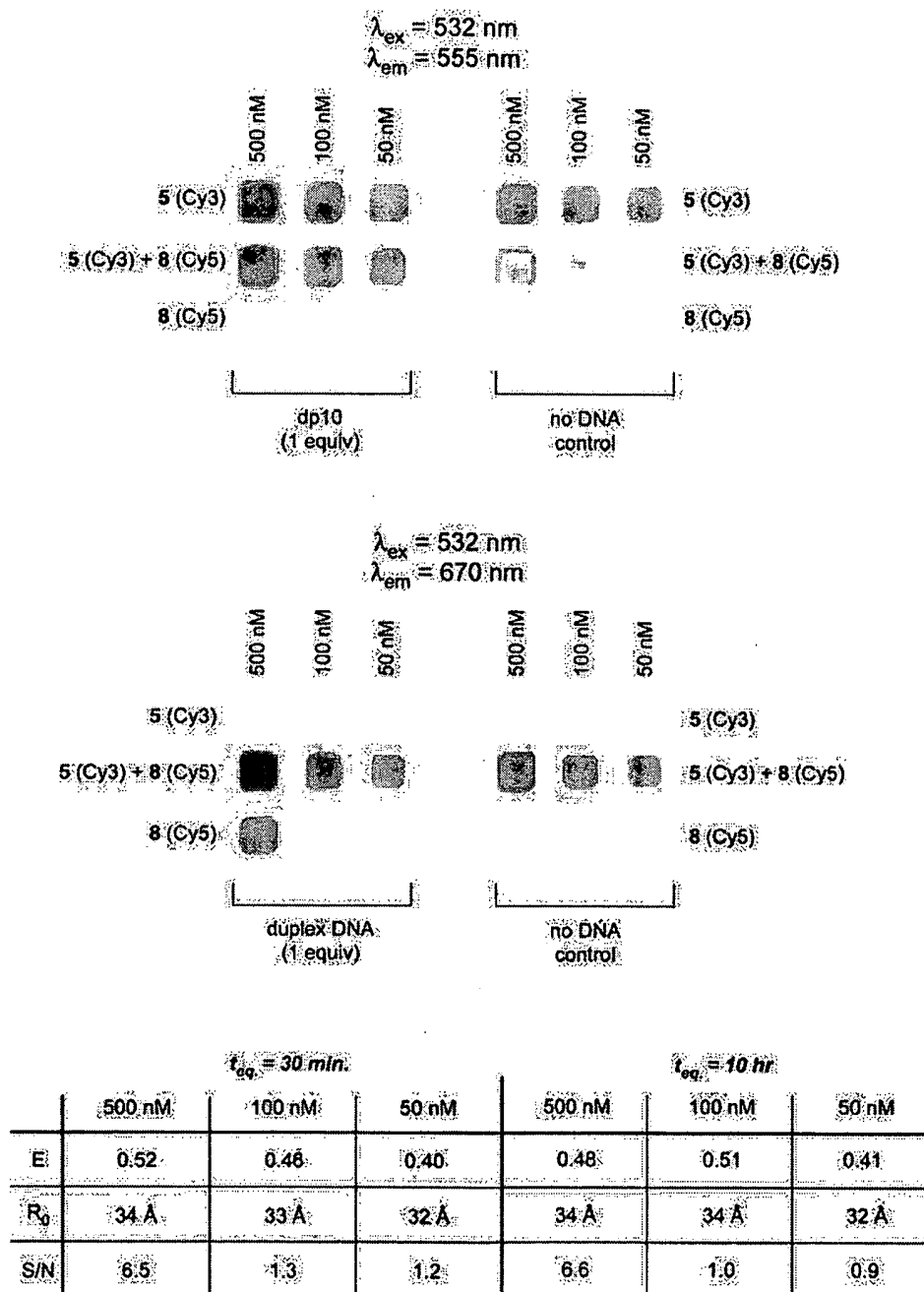


Figure 4.8 Optimization of condition for polyamide-based FRET. Microplate experimental layout and raw data is shown with emission at 555 nm indicated by red scale and emission at 670 nm shown in blue scale. Averaged data for replicate experiments is summarized in the table shown at bottom.

Future Directions for Polyamide-Based FRET

The Förster radii determined for the Cy3-Cy5 pair can be used to estimate the distance interval to which it can be applied in biological applications. Qualitative FRET measurements can usually be made in the range of $(0.2)R_0 - (0.8)R_0$, corresponding to 7-28 Å for 5 and 8. This range does allow for FRET between proximal binding sites on the double helix, and could be used to enhance the specificity of DNA detection by polyamides in genomic contexts. The non-invasive nature of polyamide association might also allow the association of proteins proximal to a polyamide binding site to be measured. Mapping of condensed DNA structures with lower S/N is another possible application.

The range of applications available to polyamide-based FRET could easily be extended by using a donor-acceptor pair with a larger R_0 . The Cy3B donor might prove useful to this end as it has even greater spectral overlap with Cy5 absorption, in addition to exhibiting a higher quantum yield and greater degree of quenching in the absence of DNA. The utility of the Cy3B-Cy5 pair can be determined using the same protocol discussed above. The Cy5Q acceptor is a quenched version of Cy5, such that FRET with Cy3 or Cy3B results in decreased quantum yield of donor without sensitized emission at the Cy5 wavelength. "Three-probe" experiments are readily adaptable single molecule experiments, where polyamide-based FRET could be measured at the donor wavelength and DNA position could be confirmed by fluorescence of an attached Cy5 fluorophore. The utility of Cy5Q can also be determined by the above protocol.

Cyanine-polyamide conjugates showed little deviation in fluorescent signal with variation in buffer conditions, suggesting applications with enzymes. Model duplexes containing an enzyme binding site flanked by polyamide binding sites could be designed to evaluate these assays. Enzymes known to cleave or bend DNA would be expected to reduce or increase FRET efficiency, respectively. The structural implications for repeat sequences or purine tracts could also be qualitatively assessed with polyamide-based FRET. Three-color experiments are also attractive as they would further enhance the specificity of detecting a given eight-ring binding site, without increasing the size of the ligand.

Conclusions

The preliminary data presented above demonstrates the potential of polyamide-based FRET as another tool for the sequence specific detection of DNA. Further work is needed to fully characterize this class of conjugates and to optimize the choice of donors and acceptors for biologically relevant applications. In this regard, the fluorescein-TMR donor-acceptor pair might prove applicable to polyamide-based experiments in live cells.

Experimental

The synthesis of cyanine-polyamide conjugates was described in detail in Chapter 2. Microplate experiments were performed as described in Chapter 3A using a Typhoon imaging system. Duplex oligonucleotides were annealed at 100 °C for 15 minutes and cooled to ambient temperature slowly before use. Concentrated stock solutions of cyanine conjugates were prepared by dissolving dry aliquots in a minimal volume of DMSO and diluting this stock with ultrapure water. Fluorescence quantum yield determinations were made relative to sulforhodamine, carboxyrhodamine, and nile blue standards obtained from Molecular Probes.

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The Glen Report

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GLEN RESEARCH

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I N S I D E

UNICAP AMIDITE

IQN

QUENCHERS

NOVEL MONOMERS

2'-FLUORO-RNA

3900 COLUMNS

INTRODUCTION

In this issue of *The Glen Report*, we are introducing a selection of new products and methodologies. One would think that capping in oligonucleotide synthesis would have been developed to the nth degree, but incomplete capping continues to be one of the main contributors to problems. We have, therefore, reviewed the capping efficiency of the current capping mixes and have compared this efficiency achieved to that achieved by a new phosphoramidite capping reagent, UniCap Phosphoramidite.

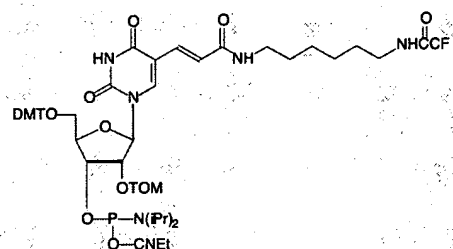
As usual, we are adding many new products to our range of nucleosides available for DNA and RNA research. As siRNA increases in significance, we have increased our focus on RNA research. We are introducing a uridine derivative (Amino-Modifier C6-U Phosphoramidite) for labeling RNA and 6-Thio-G Phosphoramidite for cross-linking to associated RNA or protein molecules. These new phosphoramidites are listed beginning on this page. We have had a long-term interest in 2'-fluoro-RNA monomers and we are happy to be able to add 2'-fluoro-A and 2'-fluoro-G to that family of products. We are also introducing our more popular supports for 3' modification on polystyrene in columns compatible with the Applied Biosystems 3900 synthesizer.

Also, we recently completed an agreement with Biosearch Technologies to begin supplying Black Hole Quencher™ (BHQ) products to the research market. These quenchers complement our current quenchers, Dabcyl and Eclipse™, and cover the complete spectral range offered by our extensive line of fluorescent dyes.

Although molecular biology products are unusual for Glen Research, we have been working recently on preparing unusual triphosphates with specific unique properties. In partnership with Lawler Scientific, LLC, we have used our chemical expertise to produce Internally Quenched Nucleotides (IQNs), which we expect will find broad acceptance in a variety of biological applications.

PRODUCTS FOR siRNA RESEARCH

FIGURE: AMINO-MODIFIER C6-U



Amino-Modifier C6-U

Amino-Modifier C6-U

Amino-Modifier C6-dT was first described^{1, 2} in the mid 1980s when interest in labeling oligos was very limited. The original usage was to attach alkaline phosphatase to oligos for diagnostic applications. The molecule was set up perfectly for this kind of use since the linker arm projects into the major groove of double-stranded DNA where there is room for large molecules without disruption of hybridization.³ Over the years, we have added products to our range based on amino-modifier C6-dT labeled with Biotin, Dabcyl, TAMRA, Fluorescein and, in this issue, BHQ-1™ and BHQ-2™. Now it is time to add the RNA analogue Amino-Modifier C6-U. Initially, we have chosen the popular TOM group⁴ for protection of the 2'-hydroxyl. We welcome Amino-Modifier C6-U to this growing structural family and envisage applications in RNA structural studies as well as for labeling siRNA to probe uptake and cellular distribution.

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UNICAP PHOSPHORAMIDITE, AN ALTERNATIVE TO ACETIC ANHYDRIDE CAPPING

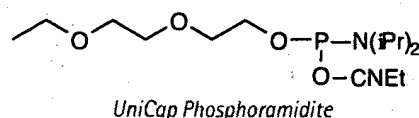
Introduction

The major impurities generated during oligonucleotide synthesis are n-1 and, to a lesser extent, n-2 deletion sequences. These deletion sequences are not homogeneous and result not from a single coupling failure but have been shown to be a statistical distribution of all possible n-1 or n-2 deletions by sequencing¹, mass spectroscopy² and hybridization to arrays of all possible complementary deletion strands.³ Deletion mutations can arise from an incomplete reaction at any of the steps during the synthesis cycle including; incomplete detritylation, incomplete capping of coupling failures or incomplete oxidation of the phosphite triester and subsequent hydrolysis back to the 5'-hydroxyl of the previous base during the next detritylation step. By far the predominant source of these deletions is incomplete capping of coupling failures during each synthesis cycle.

Standard capping is accomplished by acetylation of any remaining unreacted 5'-hydroxyls using a mix of acetic anhydride in THF (Cap A) and a capping activator, either dimethylaminopyridine (DMAP) or N-methylimidazole (Melm) in THF (Cap B). A weak organic base, either pyridine or lutidine, is added to one of the Cap mixes.

The failure to cap, and the resulting generation of excess n-1 deletion sequences, present particular problems in trityl-on purifications and in the synthesis of long oligonucleotides for gene construction and cloning. Trityl-on purification relies on the increased hydrophobicity of the trityl group which is present only on the last base or monomer unit added and not on the capped failures. Unfortunately the n-1 deletions also possess 5'-trityls which make them elute along with the full-length oligo. These trityl-on deletions can be partially eliminated in HPLC purifications by collecting only the middle of the trityl-on peak since shorter deletion oligos elute on the backside of the peak. This is not possible with cartridge purification techniques, so final oligo purity is directly dependent on the ability to efficiently cap coupling failures. Long oligos are usually purified by denaturing PAGE and n-1 deletions represent the most difficult contaminant to remove, which explains why so many long oligos used for cloning are incorrect.

FIGURE 1: UNICAP PHOSPHORAMIDITE



UniCap Phosphoramidite

In an attempt to improve overall synthesis fidelity, other approaches to capping have been explored. Since the coupling reaction is so efficient, one option is to use a phosphoramidite for capping. This option is the approach used in H-phosphonate chemistry. To that end the phosphoramidite of diethylene glycol monoethyl ether, UniCap, has been synthesized and compared to the standard capping mixes. Each capping mix was first evaluated for its ability to block oligo synthesis. Following a mock coupling using acetonitrile in place of amidite, three additional couplings were performed with the final trityl left on. This is an extreme case and represents a complete coupling failure. Quantification of the trityl-on peak represents the relative amount of capping failure. The results of these experiments conducted in quadruplicate are shown in Table 1.

The results clearly demonstrate that the capping is dependent on the activator in the Cap B solution. Melm was a less effective catalyst for acetylation with 90% capping efficiency at 10% concentration. Increasing the concentration to 16% increases capping to 97%. UniCap performs substantially better at close to 99% capping, as seen in Table 1. Although DMAP is an extremely efficient catalyst for acetylation, its use has been reported to result in modification of O⁶-dG resulting in the formation of a fluorescent adduct.⁴ For this reason DMAP has been replaced in most Cap B mixes by Melm.

Capping with UniCap Phosphoramidite

To use UniCap as a capping amidite on the Expedite 8909 or ABI synthesizers, dilute it to the standard amidite concentration and place the vial in position 5 on the instrument. ABI cycles can be modified by adding coupling steps after the last column coupling step "Column Off", replacing "Base + Tetrazole to Column" (Function 33) with "5 + Tetrazole to Column" (Function 35). For use on the Expedite synthesizer, copy the coupling steps for amidite reservoir 5 and paste them into the coupling section of each of the other amidite cycles. The standard capping steps can be left out of the cycle. Although we have been unable to confirm that acetate capping reverses any O⁶-dG modification formed during coupling, regular capping can certainly be left in the cycle if this is a concern.

When UniCap Phosphoramidite was tested, it was found to be very highly effective at nearly 99% capping efficiency and, in addition, was determined to be stable for at least one week on the synthesizer.

UniCap Phosphoramidite was originally developed for capping in oligo synthesis on the surface of chips. Capping is often omitted in this situation because acetylation by acetic anhydride can change the polarity and surface characteristics of the chips. UniCap provides virtually quantitative capping without changing the polarity of chip surfaces. This reduces the background and increases the contrast of the array fluorescence. We are grateful to Dr. Xiaolian Gao, University of Houston, for providing the following information.

TABLE 1: CAPPING EFFICIENCIES USING DIFFERENT SOLUTIONS

Synthesizer	Cap A Solution	Cap B Solution	Cap Efficiency (%)
Expedite	THF/Ac ₂ O (9:1)	10% Melm/THF/Pyr (8:1)	90.5 ± 1.9
ABI 394	THF/Pyr/Ac ₂ O (8:1:1)	10% Melm/THF	88.8 ± 2.5
ABI 394	THF/Lut/Ac ₂ O (8:1:1)	10% Melm/THF	89.1 ± 2.0
ABI 394	THF/Lut/Ac ₂ O (8:1:1)	16% Melm/THF	96.6 ± 1.4
ABI 394	THF/Lut/Ac ₂ O (8:1:1)	6.5% DMAP/THF	99.4 ± 0.3
ALL	UniCap Phosphoramidite		98.6 ± 0.4

Oligonucleotide Microarray Synthesis on Microfluidic Chip

An oligonucleotide microarray containing 3888 sequences, which are selected from human cancer related genes, was synthesized as described previously.⁵ One chip synthesis used a regular protocol with acetic anhydride (AC) capping and the other chip used the same protocol except for UniCap Phosphoramidite (PEG) capping.

DNA Chip Hybridization Using cDNA Samples

Two cDNA samples were prepared according to procedures described (<http://cmgm.stanford.edu/pbrown/protocols/index.html>). The universal (univ) and skeletal muscle (sk) total RNA was from Clontech. Fluorescent Cy3 and Cy5 dyes were incorporated using dye-dUTP for the univ and sk cDNA samples, respectively. The co-hybridization of the cDNA samples to the DNA chip used 6' SSPE (0.9 M NaCl, 60 mM Na₂HPO₄, 6 mM EDTA; pH 6.8) buffer (80 mL) mixed with 25% formamide at 32 °C for 18 h under micro-flow conditions. The chips were washed briefly with the 6' SSPE buffer before image scanning on an Axon GenePix 4000B laser scanner. The PMT level was adjusted according to the signal strength observed. The images of the AC and PEG capping DNA chips are shown in Figure 2.

Validating Oligonucleotide Synthesis on a Chip Using Hybridization

The PEG capping was implemented in DNA chip synthesis and the comparison chip was synthesized using regular AC capping. The goals in these experiments were to compare hybridization results when the two DNA chips were treated with cDNA samples labeled with Cy3 (universal total RNA sample) or Cy5 (skeletal muscle total RNA sample) fluorescent dye. The two samples were co-hybridized to the chip and the ratio of Cy3 to Cy5 is shown in color ranging from green (Cy3 > Cy5) to yellow (Cy3 = Cy5) to red (Cy5 > Cy3). The color ratio image comparison of the PEG capping versus the AC capping chip is shown in Figure 2. This experiment validates that PEG capping is applicable to DNA microarray synthesis for the improvement of capping efficiency, which is critical for the initial

AC Capping Chip



PEG Capping Chip

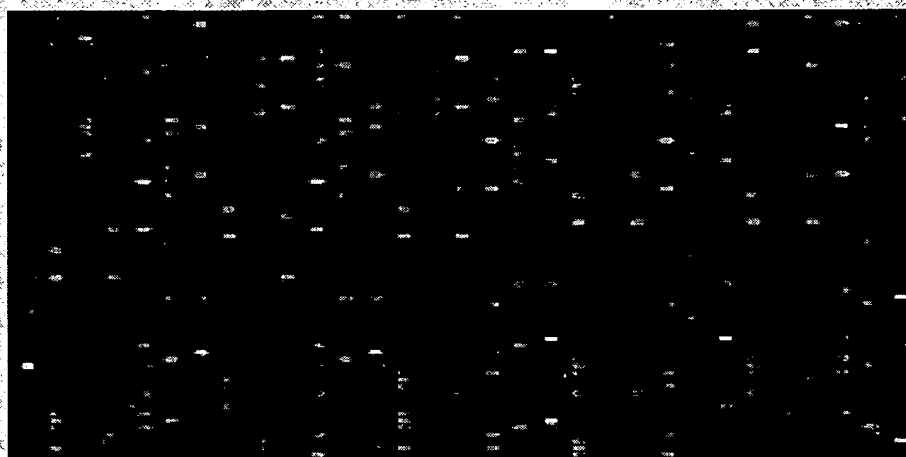


Figure 2: Images of cDNA hybridization to miniCancer Chip
Cy3(green): universal mRNA_cDNA
Cy5 (red): skeletal muscle mRNA_cDNA

synthesis steps of *in situ* oligonucleotide synthesis on glass surfaces. In addition, the capping reaction time using the PEG capping reagent is several fold shorter than that of AC capping.

The capping reagent was developed by Peilin Yu and Xiaolian Gao, Department of Chemistry, University of Houston. UniCap Phosphoramidite is sold by Glen Research under license from the University of Houston and is intended for research purposes only.

ORDERING INFORMATION

Item	Catalog No.	Pack	Price(\$)
UniCap Phosphoramidite	10-4410-02	0.25g	\$50.00
	10-4410-05	0.5g	\$100.00
	10-4410-10	1.0g	\$200.00
	10-4410-20	2.0g	\$400.00

(For Expedite vials, add "E" to catalog number.)

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EXPANDING OUR REPERTOIRE OF DARK QUENCHERS: BLACK HOLE QUENCHERS

Introduction

Fluorescence Resonance Energy Transfer (FRET) has become one of the most popular tools to assay nucleic acids. This is because FRET lends itself to high throughput automation and is quite sensitive, making it the method of choice for sequence and single nucleotide polymorphism (SNP) analysis. In addition, it is highly useful for probing DNA and RNA structure, dynamics and intermolecular interactions.

The basis of FRET is the dipole-dipole coupling of donor and acceptor molecules in which the energy of the donor in the excited state is transferred to the acceptor molecule. The efficiency of the energy transfer depends upon a variety of factors – the distance between the donor and acceptor molecules and their orientation, the quantum yield of fluorescence of the donor, the extinction coefficient of the acceptor and the spectral overlap between the emission of the donor and the absorbance of the acceptor.¹ In a traditional FRET experiment, both the acceptor and donor molecules are fluorophores, with the 5' terminus labeled with the donor and the 3' with the acceptor. Upon excitation of the donor, the acceptor fluoresces and the donor is quenched. If, however, the donor/acceptor pair is separated by a conformational change or action such as cleavage by a nuclease, the donor fluorescence is unaffected, as shown in Figure 1.

Dark Quenchers

The quencher need not be a fluorophore, however. A non-fluorescent chromophore can be used that overlaps with the donor's emission (a dark quencher). In such a case, the transferred energy is dissipated as heat.

FRET probes that utilize dark quenchers have a number of advantages over their fluorophore-labeled counterparts. They exhibit lower background fluorescence which leads to a larger signal-to-noise ratio, and, therefore, greater dynamic range.² In addition, since there is no secondary fluorescence arising from a dark quencher, multiple fluorophores can be simultaneously spectrally resolved, making dark quencher probes amenable to multiplex assays. But one of the most endearing qualities of a FRET probe designed with a dark quencher

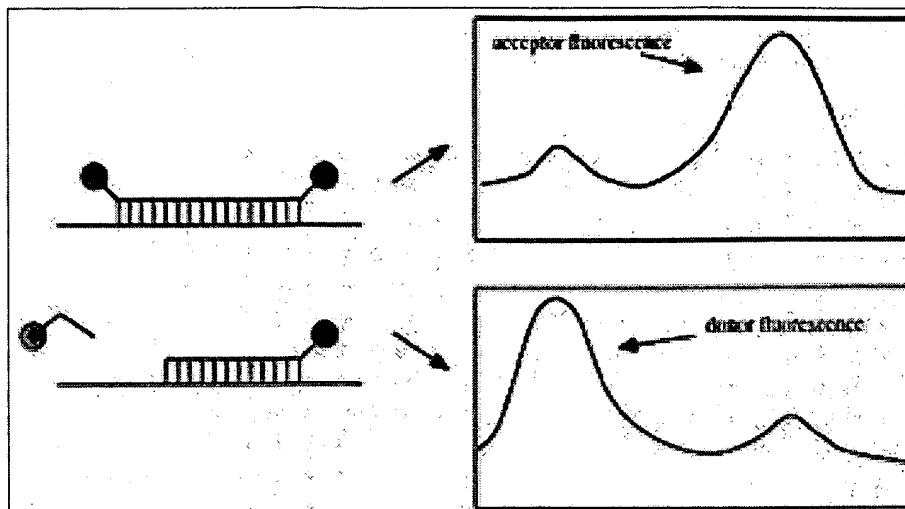


Figure 1: Schematic of FRET with a dual labeled probe before and after action of a nuclease and representative fluorescence spectra.

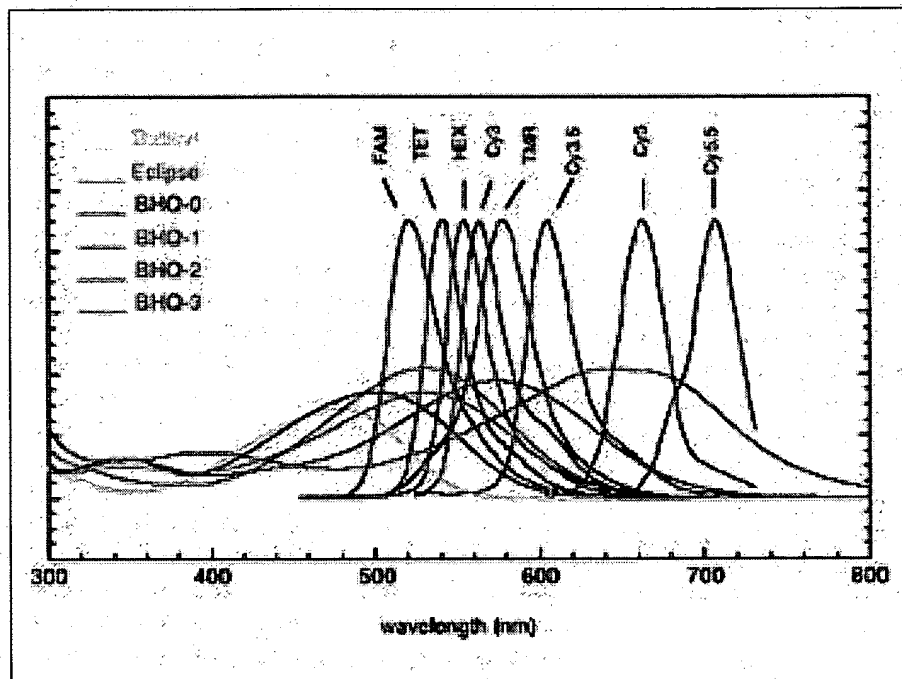


Figure 2: UV/Visible spectra of the variety of dyes and quenchers currently available from Glen Research.

TABLE 1: PHYSICAL PROPERTIES OF BLACK HOLE QUENCHERS

Quencher	λ_{max} (nm)	E_{260} (L/mol·cm)	E_{max} (L/mol·cm)
BHQ-0	493	7,700	34,000
BHQ-1	534	8,000	34,000
BHQ-2	579	8,000	38,000
BHQ-3	672	13,000	42,700

is the ease of synthesis; dark quenchers are generally more robust than their fluorescent counterparts and resist degradation during oligonucleotide deprotection. As a result, the more expensive UltraMild monomers are not required. And as an added bonus, because the failure sequences are non-fluorescent, dark quencher probes are not plagued by high background fluorescence with even unpurified probes.

One of the first reported 'dark quenchers' was the azobenzene dye Dabcyl.³ With a broad absorbance centered around 478 nm, Dabcyl was ideal for quenching dyes by FRET that fluoresce in the blue to green region, such as EDANS. However, its spectral overlap with one of the most prevalent dyes, fluorescein, was not optimal. So, in 2002, Glen Research added the Eclipse™ Quencher from Epoch Biosciences to its product line. With a maximum absorbance at 522 nm, it was ideally suited to quench fluorescein and did so at 96% efficiency.⁴

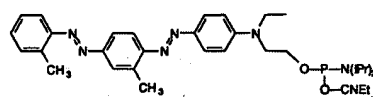
Black Hole Quencher Dyes

In keeping with the growing popularity of red and near-infrared dyes, we are expanding our dark quencher line further. We are, therefore, happy to provide in collaboration with Biosearch Technologies, the Black Hole Quencher™ dyes (BHQs), whose physical properties are detailed in Table 1. The BHQ dyes are robust dark quenchers that very nicely complement our existing product line. They are compatible with ammonium hydroxide deprotection, exhibit excellent coupling efficiencies, have large extinction coefficients and are completely non-fluorescent. Their absorbances are well-tuned to quench a variety of popular fluorophores – even those far into the red, such as Cy3 and Cy5 (Figure 2).

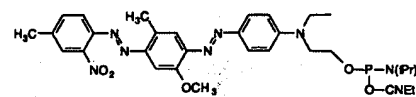
However, FRET is not the only means by which a fluorophore can be quenched. Another mechanism is static quenching due to the formation of a non-fluorescent ground-state complex. The complex is stabilized by induced-dipole and hydrophobic interactions and its formation is characterized by a decrease in the monomeric dye absorption band and an increase in a blue-shifted, non-fluorescent band. Static quenching is utilized in Molecular Beacons, in which a

FIGURE 3. BHQ STRUCTURES

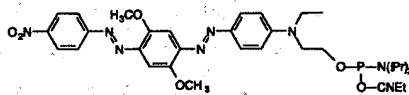
5'-BHQ Phosphoramidites



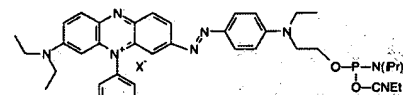
5'-BHQ-0



5'-BHQ-1

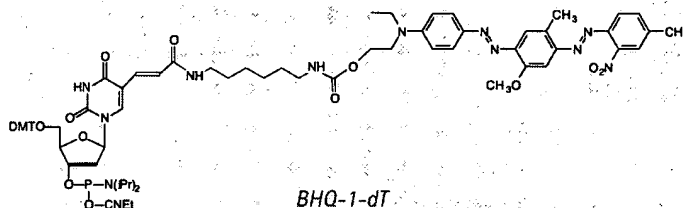


5'-BHQ-2

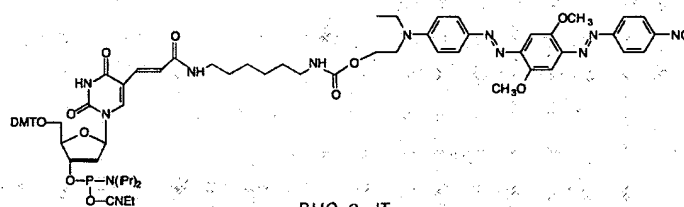


5'-BHQ-3

BHQ-dT Phosphoramidites

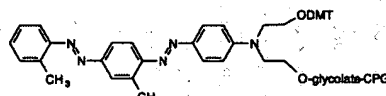


BHQ-1-dT

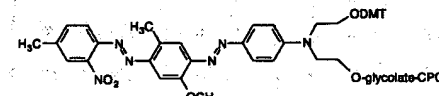


BHQ-2-dT

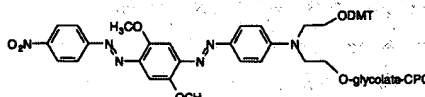
3'-BHQ CPG



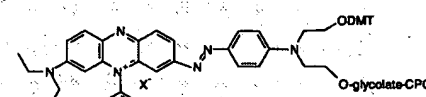
3'-BHQ-0



3'-BHQ-1



3'-BHQ-2



3'-BHQ-3

(Continued on Next Page)

dark quencher is held in close proximity to the fluorophore in a hairpin stem.⁵ The dark quencher most typically used in a Molecular Beacon is Dabcyl. Because the quenching does not involve FRET, there is little, if any, dependence upon donor-acceptor spectral overlap. However, it appears that not all dark quenchers are made equal. In a comprehensive paper by Marras, Kramer and Tyagi,⁶ the ability of BHQ-1 and BHQ-2 to quench 22 different fluorophores was evaluated. For shorter wavelength fluorophores such as fluorescein, the quenching efficiency was roughly the same as Dabcyl (91% – 93%). However, for dyes emitting in the far red, such as Cy5, the BHQ dyes were far superior – quenching the Cy5 with 96% efficiency, compared to 84% with Dabcyl. This may reflect the BHQ's ability to form stable, non-fluorescent complexes which can be a plus even in FRET probes. Indeed, recent work suggests that these non-fluorescent complexes will form even in the absence of a hairpin stem structure used by Molecular Beacons.⁷

"Black Hole Quencher", "BHQ-0", "BHQ-1", "BHQ-2" and "BHQ-3" are trademarks of Biosearch Technologies, Inc., Novato, CA. The BHQ dye technology is the subject of pending patents and is licensed and sold under agreement with Biosearch Technologies, Inc.. Products incorporating the BHQ dye moiety are sold exclusively for R&D use by the end-user. They may not be used for clinical or diagnostic purposes and they may not be re-sold, distributed or re-packaged.

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ORDERING INFORMATION

Item	Catalog No.	Pack	Price(\$)
5'-BHQ-0 Phosphoramidite	10-5930-95	50 μ mole	100.00
	10-5930-90	100 μ mole	200.00
	10-5930-02	0.25g	700.00
5'-BHQ-1 Phosphoramidite	10-5931-95	50 μ mole	100.00
	10-5931-90	100 μ mole	200.00
	10-5931-02	0.25g	700.00
5'-BHQ-2 Phosphoramidite	10-5932-95	50 μ mole	100.00
	10-5932-90	100 μ mole	200.00
	10-5932-02	0.25g	700.00
5'-BHQ-3 Phosphoramidite	10-5933-95	50 μ mole	150.00
	10-5933-90	100 μ mole	300.00
	10-5933-02	0.25g	950.00
BHQ-1-dT	10-5941-95	50 μ mole	150.00
	10-5941-90	100 μ mole	300.00
	10-5941-02	0.25g	925.00
BHQ-2-dT	10-5942-95	50 μ mole	150.00
	10-5942-90	100 μ mole	300.00
	10-5942-02	0.25g	925.00
3'-BHQ-0 CPG	20-5930-01	0.1g	190.00
	20-5930-10	1.0g	1500.00
	1 μ mole columns	Pack of 4	300.00
	0.2 μ mole columns	Pack of 4	80.00
	10 μ mole column (ABI)	Pack of 1	575.00
3'-BHQ-1 CPG	20-5930-14	Pack of 1	825.00
	20-5931-01	0.1g	190.00
	20-5931-10	1.0g	1500.00
	1 μ mole columns	Pack of 4	300.00
	0.2 μ mole columns	Pack of 4	80.00
3'-BHQ-2 CPG	20-5931-13	Pack of 1	575.00
	20-5931-14	Pack of 1	825.00
	10 μ mole column (ABI)	Pack of 1	575.00
	15 μ mole column (Expedite)	Pack of 1	825.00
	20-5932-01	0.1g	190.00
3'-BHQ-3 CPG	20-5932-10	1.0g	1500.00
	1 μ mole columns	Pack of 4	300.00
	0.2 μ mole columns	Pack of 4	80.00
	10 μ mole column (ABI)	Pack of 1	575.00
	15 μ mole column (Expedite)	Pack of 1	825.00
3'-BHQ-3 CPG	20-5932-14	Pack of 1	825.00
	20-5933-01	0.1g	190.00
	20-5933-10	1.0g	1500.00
	1 μ mole columns	Pack of 4	300.00
	0.2 μ mole columns	Pack of 4	80.00
3'-BHQ-3 CPG	20-5933-41	Pack of 4	300.00
	20-5933-42	Pack of 4	80.00
	10 μ mole column (ABI)	Pack of 1	575.00
	15 μ mole column (Expedite)	Pack of 1	825.00
	20-5933-13	Pack of 1	575.00
	20-5933-14	Pack of 1	825.00

2'-FLUORO-RNA MONOMERS

2'-Fluoro-RNA

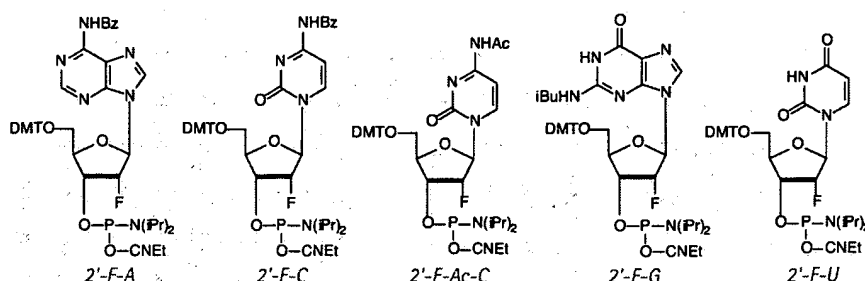
The RNA analogue, 2'-deoxy-2'-fluoro-RNA (2'-F-RNA), has always been intriguing to us and we have offered the C and U analogues for some time. Unfortunately, the A and G analogues have not been available commercially due to their extremely complex chemical syntheses.¹ We are happy to report that all four analogues are now available following production by a novel and elegant enzymatic transformation.

Fluorine has an interesting combination of properties, combining electronegativity similar to a hydroxyl group with size between an oxygen and a hydrogen atom. This combination leads to the ring of a 2'-F-ribonucleoside adopting a C3'-*endo* conformation and the resulting 2'-F-RNA oligonucleotide adopts an A-form helix on hybridization to a target. Indeed, circular dichroism (CD) spectra of 2'-F-RNA/RNA duplexes indicate that they are A-form and that the sugars have all adopted the C3'-*endo* pucker.¹ An important difference between RNA and 2'-F-RNA is that a hydroxyl group is a hydrogen bond donor while fluorine is a weak acceptor.

In studying antisense oligonucleotides, a group at Isis Pharmaceuticals¹ concluded that oligonucleotides hybridized to a target RNA oligonucleotide in the following order of increasing stability: DNA < RNA < 2'-OMe-RNA < 2'-F-RNA. With an RNA target, melting temperature (T_m) was enhanced relative to an antisense DNA oligonucleotide by 1°C per residue for RNA, 1.3 °C for 2'-OMe-RNA, and 1.8 °C for 2'-F-RNA. The stability enhancement for 2'-F-RNA hybridizing to an RNA target was additive for each 2'-F-RNA residue and slightly cooperative – i.e., the ΔT_m per substitution increases as more 2'-F-RNA residues are incorporated into the oligonucleotide. This has led to the use of 2'-F-RNA in aptamers since the resulting aptamers are not only more resistant to nucleases compared to 2'-OH RNA aptamers, but also bind ligands with higher affinities.² The use, however, of 2'-F-RNA in antisense applications is limited since the 2'-F-RNA exhibits little enhanced nuclease resistance compared to DNA and its hybrid duplex does not activate RNase-H. Interestingly, 2'-F-RNA can be used quite effectively in siRNA applications.

Recent work by Layzer et al., demonstrated that siRNA synthesized with

FIGURE 1: 2'-FLUORO-RNA MONOMERS



2'-F pyrimidines showed greatly increased stability in human plasma compared to 2'-OH siRNA.³ They were functional in cell culture and *in vivo* using BALB/c mice transfected with pGL3 luciferase. Interestingly, though the 2'-F siRNA was significantly more stable than 2'-OH siRNA, they were only slightly more inhibitory over time in cell culture than 2'-OH siRNA; *in vivo*, their activities were practically the same. The authors note that these results may depend upon the siRNA delivery methodology.

Less has been reported on the stability of duplexes between 2'-F-RNA and DNA. In a study⁴ of the cleavage of RNA/DNA duplexes by RNase H, 2'-F-Adenosine (2'-F-A) oligonucleotides and chimeras containing 2'-F-A and rA were used to evaluate the ability of the modified RNA strand to promote varying levels of RNase-H activity. The authors measured the T_m of 18-mer oligonucleotides containing rA and/or 2'-F-A to oligo-T18 and found that the homopolymer of 2'-F-A enhanced binding by 0.5° per residue relative to rA. However, chimeras of 2'-F-RNA and rA were unpredictable in their melting behavior and some actually lowered the duplex T_m .

Our own melting experiments of duplexes containing 2'-F-RNA supported these results. We have found that a single substitution of 2'-F-RNA in a mixed base DNA/DNA dodecamer increased the T_m by 1.2 °C. However, further substitutions with two or four 2'-F-RNA residues led to a drop in the T_m by 1.3 °C. Interestingly, a fully substituted 2'-F-RNA/DNA duplex does exhibit higher stability, with the T_m being increased by 0.5° per incorporation.

With a full set of monomers now available, we predict applications for 2'-F-RNA in ribozymes, siRNA and structural DNA research. By making all four monomers available, we hope to open up a full spectrum of research applications for 2'-F-RNA.

References:

- (1) A.M. Kawasaki, et al., *Journal of Medicinal Chemistry*, 1993, **36**, 831-841.
- (2) M. Khati, M. Schuman, J. Ibrahim, Q. Sattentau, S. Gordon, and W. James, *J Virol*, 2003, **77**, 12692-8.
- (3) J.M. Layzer, A.P. McCaffrey, A.K. Tanner, Z. Huang, M.A. Kay, and B.A. Sullenger, *RNA*, 2004, **10**, 766-71.
- (4) D.R. Yazbeck, K.L. Min, and M.J. Damha, *Nucleic Acids Res.*, 2002, **30**, 3015-25.

ORDERING INFORMATION

Item	Catalog No.	Pack	Price(\$)
2'-F-A-CE Phosphoramidite	10-3400-90	100 µmole	235.00
	10-3400-02	0.25g	650.00
2'-F-C-CE Phosphoramidite	10-3410-90	100 µmole	165.00
	10-3410-02	0.25g	500.00
2'-F-Ac-C-CE Phosphoramidite	10-3415-90	100 µmole	165.00
	10-3415-02	0.25g	500.00
2'-F-G-CE Phosphoramidite	10-3420-90	100 µmole	235.00
	10-3420-02	0.25g	650.00
2'-F-U-CE Phosphoramidite	10-3430-90	100 µmole	165.00
	10-3430-02	0.25g	500.00

INTERNALLY QUENCHED NUCLEOTIDE FLUORESCENT REPORTERS

Joseph F. Lawler, Jr.
Lawler Scientific, LLC

Introduction

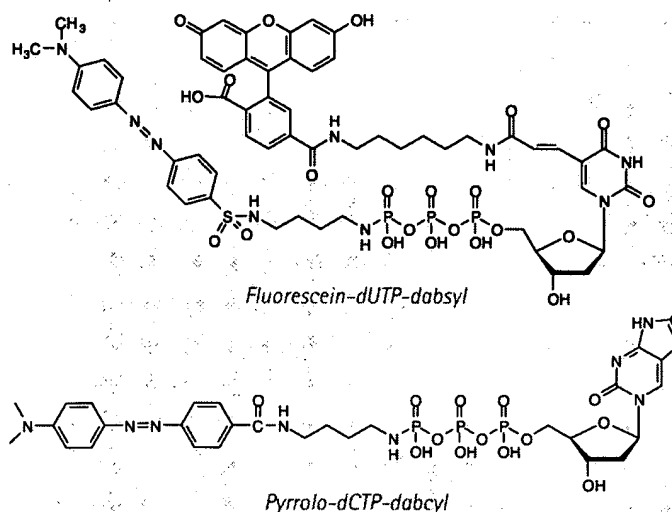
Several methods have been developed for enzymatic fluorescent labeling of nucleic acids. A dNTP analog can be used to incorporate a fluorophore by PCR, nick translation or random priming, either directly into DNA¹ or indirectly via a hapten such as biotin.² Though high incorporation efficiencies have been reported,³ all of these approaches require the separation of unincorporated label prior to downstream applications.

Lawler Scientific, LLC has developed a series of reagents called Internally Quenched Nucleotides or IQNs. These reagents consist of a nucleoside triphosphate with a fluorescent reporter attached to the nucleobase and a quencher moiety attached to the gamma-phosphate. The nucleotides remain non-fluorescent until the quencher is enzymatically separated from the parent nucleotide. Since the IQNs are non-fluorescent until incorporated into a nucleic acid, they should not give rise to the background fluorescence signals commonly observed when DNA labeled by standard means is inadequately purified.

Nucleic Acid Labeling

The first generation IQN consists of a fluorescein-dUTP with a dabsyl quencher linked to the gamma phosphate (see Figure

FIGURE 1: STRUCTURES OF INTERNALLY QUENCHED NUCLEOTIDES (IQNs)



1). The first generation molecules were developed to address the cDNA labeling application described above. Fluorescein and dabsyl were selected because of their superior optical properties and because the photophysics governing their interaction is well described in the literature.⁴ In addition, this IQN is soluble and stable in aqueous solution.

Molecular modeling of the fluorescein and dabsyl moieties of the IQN predicts that these moieties will be well within effective quenching distances. Consistent with this prediction is the observation that fluorescence emission of this first

generation molecule is >98% quenched (see Figure 2). Chemical fragmentation of the triphosphate backbone restores fluorescence emission as does hydrolysis by snake venom phosphodiesterase (SVP). As shown in the accompanying Figure 3, SVP rapidly hydrolyzes the IQN with a concomitant increase in fluorescence intensity at 520nm.

Reverse transcriptases are commonly used in cDNA labeling protocols. An oligonucleotide primer extension assay was performed using Avian Myeloblastosis Virus (AMV) reverse transcriptase (RT). AMV RT correctly incorporated the IQN opposite dA

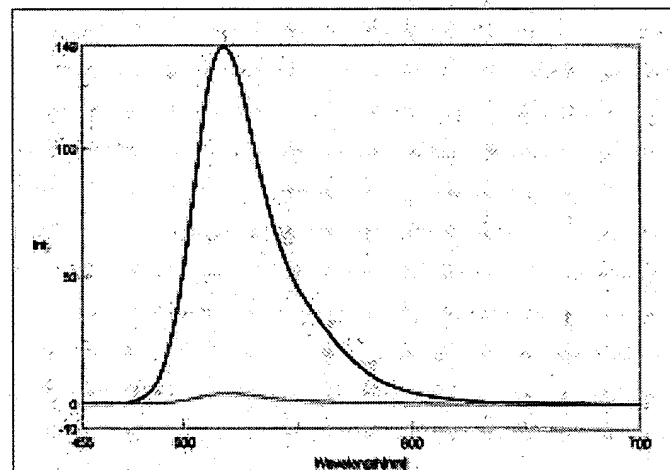


Figure 2: The fluorescence spectrum of equimolar amounts of Fluorescein-dUTP (black) and Fluorescein-dUTP-dabsyl (red) were recorded at an excitation wavelength of 440nm.

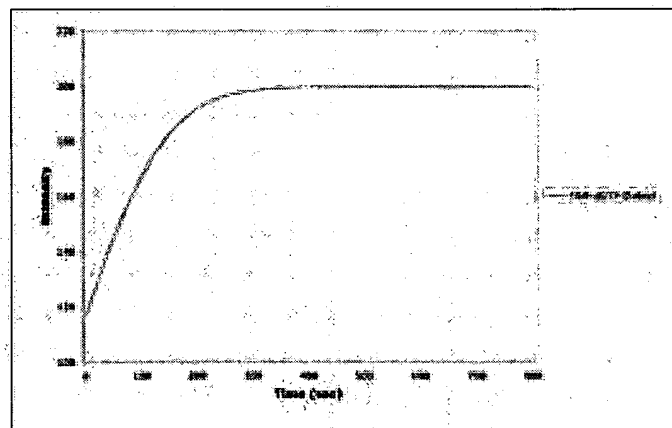


Figure 3: The hydrolysis of fluorescein-dUTP-dabsyl by snake venom phosphodiesterase. Note: The background fluorescence was essentially zero prior to the addition of the SVP.

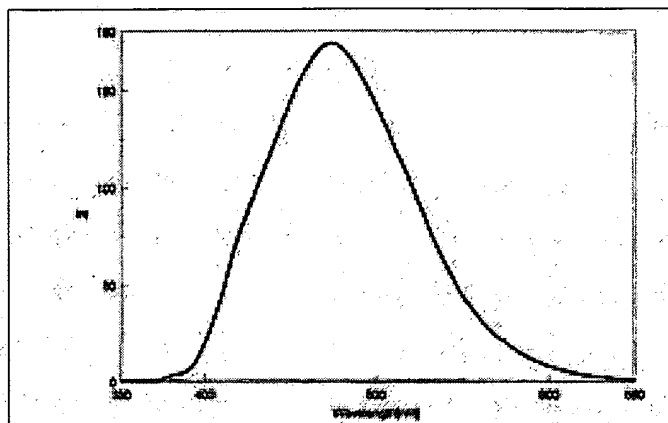


Figure 4: The fluorescence spectrum of equimolar amounts of Pyrrolo-dCTP (black) and Pyrrolo-dCTP-dabcyl (red) were recorded at an excitation wavelength of 340nm.

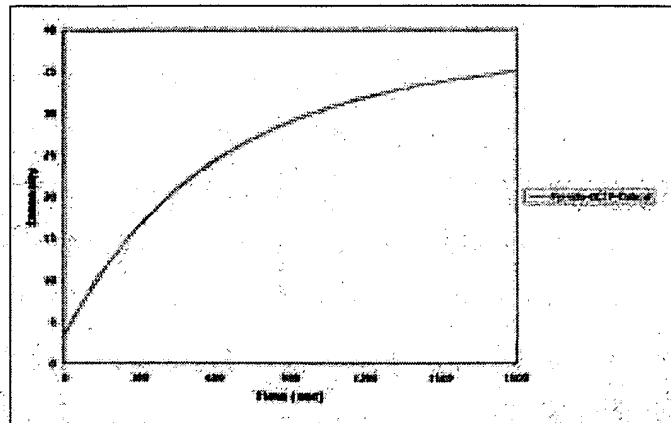


Figure 5: The hydrolysis of pyrrolo-dCTP-dabcyl by snake venom phosphodiesterase.

residues in the template strand. In a second set of experiments, this IQN was added to an AMV RT mediated cDNA synthesis reaction. The addition of the IQN resulted in label incorporation in the resulting 351nt cDNA. cDNA synthesis reactions can therefore be "doped" with IQNs to achieve the desired amount of label incorporation.

The IQN technology may extend microarray and real-time PCR techniques but it would do so in different ways. Microarray hybridization experiments may be made more time- and cost-efficient by eliminating the fluid handling steps associated with the cDNA labeling and purification processes. Real-time PCR based experiments may also be made more time- and cost-efficient by eliminating the empiric and costly process of designing and testing fluorescent quantification probes.

Real time PCR

Lawler Scientific, LLC also recognized the potential utility of these reagents for monitoring nucleic acid amplification reactions in real time. Several probe based methods have been developed for quantifying PCR. While effective, these methods require the design and synthesis of an oligonucleotide for each amplicon of interest. Since the use of IQNs is independent of the amplicon chosen, IQNs can serve as an "off the shelf" reagent for monitoring the progress of a PCR.

Thermophilic DNA polymerases tend to be more stringent than reverse

transcriptases with respect to substrate specificity. Using an extensive body of structure activity relationship data, Lawler Scientific, LLC developed a less extensively decorated IQN for use in real-time PCR. A second generation IQN was prepared using pyrrolo-dC, an intrinsically fluorescent nucleobase developed at Glen Research,⁵ as a fluorophore and dabcyl as a quencher (see Figure 1).

Again, the fluorescence emission of pyrrolo-dCTP-dabcyl is >98% quenched (Figure 4) and hydrolysis by SVP leads to the return of fluorescence (Figure 5). The absorbance and emission spectra of pyrrolo-dC allow it to be detected in the presence of other nucleobases and nucleic acids. In addition, pyrrolo-dC is known⁵ to be incorporated opposite dG positions by *Taq* DNA polymerase.

Other Applications

Lawler Scientific, LLC is commercializing several additional IQNs with varying fluorophore and quencher pairs. It is

anticipated that these molecules will be useful in multiple-color hybridization reactions, SNP analysis, and polymerase detection.

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References:

- (1) H. Yu, J. Chao, D. Patek, R. Mujumdar, S. Mujumdar, and A.S. Waggoner, *Nucleic Acids Res.*, 1994, **22**, 3226-32.
- (2) X. Li, W.M. James, F. Traganos, and Z. Darzynkiewicz, *Biotech Histochem*, 1995, **70**, 234-42.
- (3) T. Tasara, et al., *Nucleic Acids Res.*, 2003, **31**, 2636-46.
- (4) S.A.E. Marras, F.R. Kramer, and S. Tyagi, *Nucleic Acids Res.*, 2002, **30**, E122.
- (5) D.A. Berry, et al., *Tetrahedron Lett*, 2004, **45**, 2457-2461.

ORDERING INFORMATION

Item	Catalog No.	Pack	Price(\$)
Fluorescein-dUTP-dabsyl 1mM, 25nmoles, 10mM Tris, 1mM EDTA	88-1056-01	25 µL	\$270.00
Pyrrolo-dCTP-dabcyl 1mM, 25nmoles, 10mM Tris, 1mM EDTA	88-1017-01	25 µL	\$270.00

COLUMNS

AB 3900 and MerMade Columns

We are happy to announce the availability of some of our more popular minor base supports on polystyrene and in columns fully compatible with the Applied Biosystems 3900 synthesizer. These include our popular Universal Support II, which will allow oligos to be produced on the 3900 with ANY base at the 3' terminus. At the

same time, we are offering 1 μ mole columns for the 3900 instrument.

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ORDERING INFORMATION

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AB 3900			
US II PS			
200 nmole columns	26-5110-52	Pack of 10	100.00
40 nmole columns	26-5110-55	Pack of 10	100.00
3'-Phosphate PS			
200 nmole columns	26-2900-52	Pack of 10	150.00
40 nmole columns	26-2900-55	Pack of 10	150.00
3'-PT-Amino-Modifier C6 PS			
200 nmole columns	26-2956-52	Pack of 10	220.00
40 nmole columns	26-2956-55	Pack of 10	220.00
3'-(6-FAM) PS			
200 nmole columns	26-2961-52	Pack of 10	300.00
40 nmole columns	26-2961-55	Pack of 10	300.00
3'-Dabcyl PS			
200 nmole columns	26-5912-52	Pack of 10	300.00
40 nmole columns	26-5912-55	Pack of 10	300.00
3'-TAMRA PS			
200 nmole columns	26-5910-52	Pack of 10	300.00
40 nmole columns	26-5910-55	Pack of 10	300.00
3'-BiotinTEG PS			
200 nmole columns	26-2955-52	Pack of 10	300.00
40 nmole columns	26-2955-55	Pack of 10	300.00
dA PS 1 μ mole columns	26-2600-61	Pack of 200	927.00
dC PS 1 μ mole columns	26-2610-61	Pack of 200	927.00
dmf-dG PS 1 μ mole columns	26-2629-61	Pack of 200	927.00
dT PS 1 μ mole columns	26-2630-61	Pack of 200	927.00
96 Well Format			
Universal Support 1000			
200 nmole columns	20-5001-92	Pack of 96	250.00
40 nmole columns	20-5001-95	Pack of 96	250.00
Universal Support II			
200 nmole columns	20-5110-92	Pack of 96	250.00
40 nmole columns	20-5110-95	Pack of 96	250.00

(Continued from Front Page)

Amino-Modifier C6-U (cont.)

- (3) J. Telser, K.A. Cruickshank, L.E. Morrison, and T.L. Netzel, *J. Am. Chem. Soc.*, 1989, **111**, 6966-6976.
- (4) S. Pitsch, P.A. Weiss, L. Jenny, A. Stutz, and X.L. Wu, *Helv Chim Acta*, 2001, **84**, 3773-3795.

6-Thio-G

Nucleotides with sulfur substituting for oxygen at the 4-position of pyrimidines and the 6-position of purines have proved to be very useful in molecular biology. In particular, the sulfur is active photochemically and available to photo-crosslink to an adjacent molecule, allowing study of internucleotide and nucleotide-protein interactions. Because the sulfur is at a position actively used for hydrogen bond interactions, the distance to the complementary target is short and cross-linking occurs specifically, allowing internucleotide interactions to be explicitly defined. Sulfur analogues of 2'-deoxynucleosides have been available as phosphoramidites for a long time and 4-thiouridine (4-thio-U) in the RNA series has been available also. We now add 6-thioguanosine (6-thio-G) to the tools available for studying RNA-RNA and RNA-protein interactions by offering 6-thio-G phosphoramidite for incorporation into oligoribonucleotides.

There have been several reports¹⁻³ in the literature describing 6-thio-G phosphoramidite but it is only recently that the demand for minor RNA phosphoramidites has made this feasible as a product. It is easy to envisage applications for this product in ribozyme and siRNA applications, as well as in RNA-protein interactions.

The removal of the silyl protecting group without interfering with the sulfur is critical, so we have used the more traditional t-butyldimethylsilyl protecting group on the 2'-hydroxyl. This is removed cleanly by triethylamine trihydrofluoride in DMSO but t-butylammonium fluoride (TBAF) leads to degradation of the thio-nucleotide analogue and should not be used.

References:

- (1) C.J. Adams, J.B. Murray, M.A. Farrow, J.R.P. Arnold, and P.G. Stockley, *Tetrahedron Lett.*, 1995, **36**, 5421-5424.

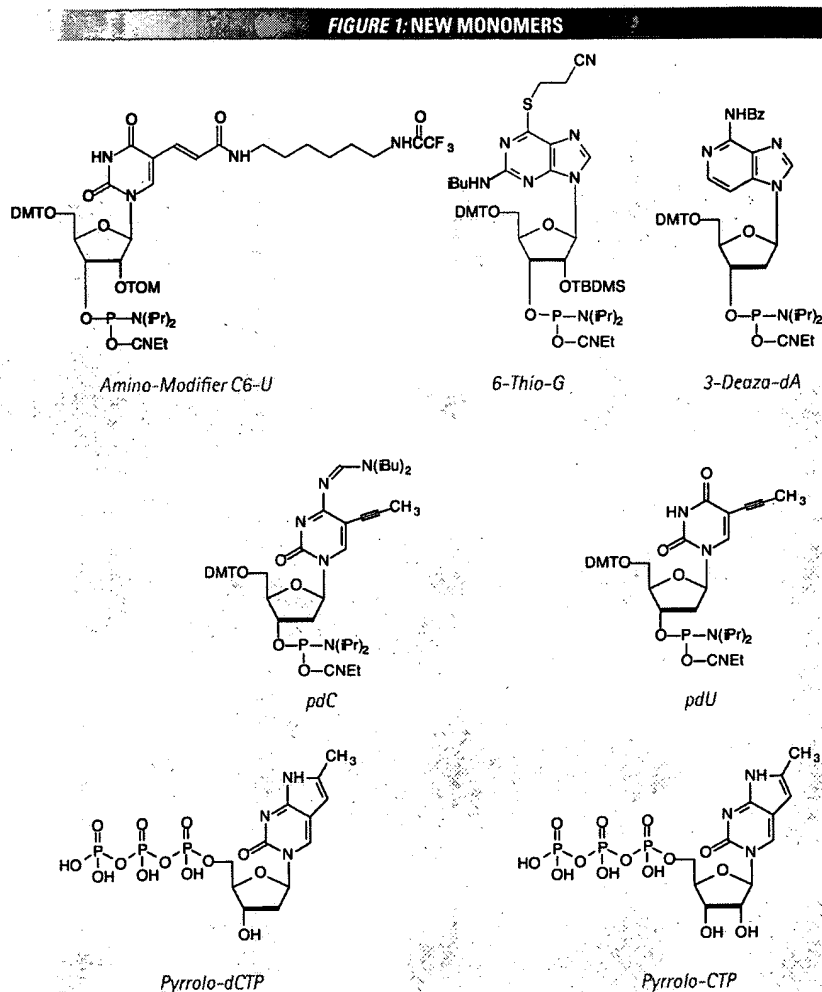
- (2) Y. Wang, E. Lattmann, and Q. Zheng, *Nucleos Nucleot Nucleic Acids*, 2003, **22**, 1247-1249.
 (3) Q.G. Zheng, Y. Wang, and E. Lattmann, *Bioorg Medicinal Chem Letter*, 2003, **13**, 3141-3144.

3-Deaza-dA

Modified base analogues of 2'-deoxynucleosides are readily available for probing interactions in the major groove of duplex DNA. However, there are far fewer analogues available to investigate interactions in the minor groove. The standard nucleobases have an unshared pair of electrons that project into the minor groove of duplex DNA. In the case of the purines, this is the nitrogen at N³ and, for the pyrimidines, it is the keto group at C². Enzymes that interact with DNA, polymerases, reverse transcriptases, restriction enzymes, etc., may use a hydrogen bond donating group to contact the hydrogen bond acceptor in the minor groove. 3-Deaza-2'-deoxyadenosine is very interesting in that it maintains the ability for regular Watson-Crick hydrogen bonding to T but is lacking the electron pair at the 3-position normally provided by N³. A very interesting recent publication from the Benner group describes¹ using 3-deaza-2'-deoxyadenosine to probe minor groove contacts by polymerases and reverse transcriptases in the context of biological evolution.

An earlier paper² discussed the thermodynamic stability of oligonucleotides containing 3-deaza-2'-deoxyadenosine. Surprisingly, substitution of 3-deaza-2'-deoxyadenosine for 2'-deoxyadenosine lowered duplex stability substantially by around 4° per insertion. The authors surmised that this was due to the higher pK_a (6.80) of 3-deaza-2'-deoxyadenosine in comparison to 2'-deoxyadenosine (3.62), which allowed protonation of the base, and the loss of stabilizing hydration of the minor groove electron pair.

We have had a long-term interest in supplying the phosphoramidite of 3-deaza-2'-deoxyadenosine, which was very challenging^{3,4} to prepare in quantity. We are delighted to offer this phosphoramidite as a result of the perseverance of our colleagues at Berry and Associates, Inc.



References:

- (1) C.L. Hendrickson, K.G. Devine, and S.A. Benner, *Nucleic Acids Res.*, 2004, **32**, 2241-50.
- (2) C. Lever, X. Li, R. Cosstick, S. Ebel, and T. Brown, *Nucleic Acids Res.*, 1993, **21**, 1743-1746.
- (3) R. Cosstick, X. Li, D.K. Tuli, D.M. Williams, B.A. Connolly, and P.C. Newman, *Nucleic Acids Res.*, 1990, **18**, 4771.
- (4) F. Seela, T. Grein, and S. Samnick, *Helvetica Chimica Acta*, 1992, **75**, 1639-1650.

C-5 propynyl Pyrimidine Analogues

Substitution of C-5 propynyl-dC (pdC) for dC and C-5 propynyl-dU (pdU) for dT¹ are effective strategies to enhance base pairing. This increase in hybridization efficiency is due to the hydrophobic nature of the groups at the C-5 position which helps to exclude water molecules from the duplex.

Using these base substitutions, duplex stability and therefore melting temperatures

are raised by the approximate amounts shown:

C-5 propynyl-C	2.8° per substitution.
C-5 propynyl-U	1.7° per substitution

While these modifications have found most applications in antisense oligonucleotides, their ability to enhance binding while maintaining specificity will also prove useful in the synthesis of high affinity probes.

At the time of writing, we are completing an agreement with Isis Pharmaceuticals, Inc. to recommence the supply of these two valuable products. We believe these will be a welcome addition to the selection of modified bases that affect hybridization.

References:

- (1) B.C. Froehler, S. Wadwani, T.J. Terhorst, and S.R. Gerrard, *Tetrahedron Lett.*, 1992, **33**, 5307-5310.

Pyrrolo-CTP

Pyrrolo-dC is a fluorescent nucleoside that codes as dC and base pairs efficiently with dG. We have published a preliminary report¹ on the chemistry of pyrrolo-dC and further details of the chemistry and biology are currently in press. Preliminary evidence indicates that pyrrolo-dC triphosphate is an excellent substrate for Taq, Pfu and Vent polymerases and is incorporated specifically opposite dG. Pyrrolo-dCTP has been available for some time and is in use in biological assays. We are now introducing pyrrolo-CTP, the ribonucleoside triphosphate. We anticipate that the addition of a fluorescent ribonucleotide with fluorescence exquisitely sensitive to its environment would be of great interest for RNA structural research.

The pyrrolo-C project is a joint development by Berry and Associates (<http://www.berryassoc.com>) and Glen Research.

ORDERING INFORMATION

Item	Catalog No.	Pack	Price(\$)
Amino-Modifier C6-U Phosphoramidite	10-3039-95	50 μ m	360.00
	10-3039-90	100 μ m	720.00
	10-3039-02	0.25g	1475.00
6-Thio-G-CE Phosphoramidite	10-3072-95	50 μ m	250.00
	10-3072-90	100 μ m	500.00
	10-3072-02	0.25g	1200.00
3-Deaza-dA-CE Phosphoramidite	10-1088-95	50 μ m	177.50
	10-1088-90	100 μ m	355.00
	10-1088-02	0.25g	975.00
Pyrrolo-dCTP 10mM	81-1017-01	100 μ L	\$150.00
Pyrrolo-CTP 10mM	81-3017-01	100 μ L	\$270.00

Patents covering this modified fluorescent base and its uses are currently pending.

References:

- (1) D.A. Berry, et al., *Tetrahedron Lett*, 2004, **45**, 2457-2461.



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